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PRINCIPAL INVESTIGATOR: Christopher M. Heaphy, B.S.
Jeffrey K. Griffith, Ph.D.

CONTRACTING ORGANIZATION: University of New Mexico
Health Sciences Center
Albuquerque, NM 87131-5221

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Christopher M. Heaphy, B.S. and Jeffrey K. Griffith, Ph.D.

E-Mail: cheaphy@salud.unm.edu

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14. ABSTRACT: We have shown in two independent retrospective studies that loss of telomere content (TC) has potential value in predicting clinical outcome in breast cancer. However, an alternative marker for TC, which could be assessed in samples with small numbers of cells, such as fine needle aspirates, with commonly used methods is desirable. The aim of this study is to demonstrate that measurement of allelic imbalance (AI), which could be easily adapted to the clinical laboratory setting, can serve as a surrogate for TC, discriminating between women in need of more aggressive treatment and those for whom aggressive protocols are unnecessary. The candidate has developed a robust assay to determine the extent of AI that discriminates between normal and tumor specimens with 67% sensitivity and 99% specificity. Currently, the candidate is assessing the potential prognostic capabilities of the assay in node negative breast tumors. Additionally, the candidate has shown that increased AI and altered TC are present in both tumors and surrounding histologically normal breast tissues at distances at least one 1cm from the visible tumor margins and decrease as a function of distance. In addition to evaluating a potential biomarker of breast cancer progression, the proposed investigation has provided the candidate opportunities to interact with pathologists and oncologists to learn normal and abnormal breast morphology, the strengths and limitations of currently used breast cancer biomarkers and the scientific rationale for ongoing clinical trials. To date, all tasks, as outlined in the Statement of Work, are on schedule.

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I. INTRODUCTION

Our laboratory has shown in two independent retrospective studies that loss of telomere content (TC), a surrogate for telomere length, has potential value in predicting clinical outcome in breast cancer. While TC appears to provide a sensitive predictor of disease-free survival in women with breast cancer, an alternative marker for TC, which could be assessed in samples with small numbers of cells, such as fine needle aspirates, with commonly used methods, such as PCR, is desirable. The aim of this study is to demonstrate that measurement of allelic imbalance (AI), which could be easily adapted to the clinical laboratory setting, can serve as a surrogate for TC, discriminating between women in need of more aggressive treatment and those for whom aggressive protocols are unnecessary. In addition to evaluating a potential biomarker of breast cancer progression, the proposed investigation will provide the candidate opportunities to interact with pathologists and oncologists to learn normal and abnormal breast morphology, the strengths and limitations of currently used breast cancer biomarkers, current standards of breast cancer treatment and the scientific rationale for ongoing clinical trials. To date, all tasks, as outlined in the Statement of Work, are on schedule.

Hypothesis and Rationale

Our preliminary results suggest that the extent of AI may have prognostic value in breast cancer. Consistent with this notion, Kronenwett and colleagues have shown that the degree of genomic instability allows additional classifying of the known aneuploid, diploid, and tetraploid categories of primary breast adenocarcinomas into low and high malignant subtypes. Therefore, *we hypothesize that measuring the extent of AI at diverse microsatellite loci provides a global assessment of overall genomic instability in a tumor and its surrounding microenvironment and has value in predicting breast cancer progression*. To test this hypothesis we will assess the potential prognostic value of AI in human breast tumor samples. Additionally, we propose to study AI in coexisting histologically normal (CHN) breast tissue and in stromal and epithelial cell populations. This hypothesis will be evaluated through three specific aims.

- **Specific Aim #1:** *To assess the potential use of allelic imbalance in predicting disease-free survival by conducting a retrospective study on node negative breast tumors.*

- **Specific Aim #2:** *To assess the extent of allelic imbalance as a function of distance from tumor margins in breast tumors and co-existing histologically normal breast tissue, to determine if stromal and epithelial cells display different patterns of allelic imbalance, and to identify molecular signatures associated with the extent of allelic imbalance in stromal and epithelial cells.*

- **Specific Aim #3:** *To compare the extent of allelic imbalance to pathological grading in invasive breast tumors by conducting a prospective study on breast tumors.*

II. KEY ACCOMPLISHMENTS

IIa. RESEARCH ACCOMPLISHMENTS

During the first two years of this training grant, I have accomplished the following:

- Unbalanced allelic loci and altered telomere length are present in both tumors and surrounding CHN breast tissues at distances at least one centimeter from the visible tumor margins and decrease as a function of distance. Additionally, unbalanced loci are conserved between the surrounding breast tissues and the tumors, implying cellular clonal evolution (Appendix A).

- Determined that TC in a cohort of breast cancer tissues (N=77) predicts breast cancer-free survival interval independent of age at diagnosis and TNM stage, and may discriminate by stage (Appendix B). This finding was confirmed in a larger, population-based study (N=530), where TC predicted overall survival interval. Additionally, TC predicted breast cancer-free survival interval in this group independent of TNM stage, p53 status and ER status (Appendix C).

- Measured AI in normal and tumor specimens from varying organs and determined that the AI method, developed by the candidate, is able to discriminate between normal and tumor specimens with 67% sensitivity and 99% specificity (Appendix D).

- Validated findings in the breast by performing a study in the prostate, another hormone-dependent organ. In a retrospective analysis, TC measured in prostate biopsy tissue (N=103) predicts early likelihood of post-prostatectomy PSA recurrence independent of pre-operative PSA level and biopsy Gleason sum, particularly in men with Gleason sum ≤ 7 disease (Appendix E).

- Identified transcripts by microarray technology that were significantly over expressed, relative to normal breast tissue, in patient-matched, tumor-adjacent, histologically normal (TAHN) tissues obtained from sites 1 cm (N=6) and 5 cm (N=6) from the visible tumor margin (TAHN-1 and TAHN-5 tissues, respectively) and confirmed expression in a set of reduction mammoplasty (RM) breast tissues (N=5). We also demonstrated that a subset of these genes is selectively over expressed in TAHN-1 tissues, including collagens alpha 1(I), 1(III) and 2 (I). This is particularly provocative, as increased collagen synthesis is indicative of reactive stroma, which may act as a supportive agent in tumorigenesis. The existence of a subset of genes selectively over expressed in TAHN-1 tissues suggests that there is a gradient of selective, differential gene expression extending out from the tumor margin (Appendix F).

IIb. TRAINING/EDUCATIONAL ACCOMPLISHMENTS

Since the activation of this award, the Ph.D. candidate has been provided the opportunity to work and interact with oncologists, surgeons, pathologists and other scientists who all specialize in breast cancer. The candidate has attended journal clubs, specialized departmental and Cancer Center seminars and has been an active participant in the Breast Cancer Working Group. The candidate's research is overseen by his dissertation committee, a group comprised of three Ph.D. scientists with interests in breast cancer, and one M.D. who specializes in breast cancer pathology.

On the educational level, the candidate has helped instruct three upper-level Biochemistry courses: (1) Biochemical Methods Laboratory (2) Intensive Biochemistry I (3) Intensive Biochemistry II: Intermediary Metabolism. Additionally, the candidate is currently a teaching assistant for a graduate level cancer biology course. The candidate plans on continuing his breast cancer research in an academic setting, thus these teaching experiences will provide him with the necessary teaching skills to further his career.

IIc. PERFORMANCE ACCOMPLISHMENTS

Experimental Milestones

Specific Aim 1 (4 tasks)

Task 1 Month 1-12 **Completed**

- o Identify and procure archival specimens from the New Mexico Tumor Registry (NMTR) at the University of New Mexico School of Medicine based on patient recurrence status.
- In year one, 184 node negative breast tumors were procured from NMTR.
- In year two, an additional set of 312 node negative breast tumors were obtained.

Task 2 Months 12-14 **Completed**

- o Isolate DNA from the paraffin-embedded breast tumors.
- In year one, DNA was isolated from all 184 collected specimens.
- In year two, DNA was isolated from the additional set of 312 cases.

Task 3 Months 14-24 **Completed**

- o Measure AI in the paraffin-embedded breast tumors.
- In year one, AI was successfully determined in 172 of the 184 collected samples.
- In year two, AI was successfully determined in 280 of the 312 additionally collected specimens.

Task 4 Months 24-30 **In Progress**

- o Analyze the correlation between the AI and patient recurrence status.
- In year two, the analysis of the correlation between the extent of AI and patient recurrence status was initiated. However, due to a limited number of recurrent cases, 30 additional cases with a documented breast cancer recurrence are needed for determination of significant results.

Specific Aim 2 (7 tasks)

Task 1 Months 1-30 **In Progress**

- o Prospectively, collect mastectomies and CHN breast tissues (1cm and 5cm from visible tumor margins).
- In year one, 17 cases (tumor, 1cm, 5cm tissues) were collected.
- In year two, an additional 11 cases were prospectively collected.

Task 2 Months 6-32 **In Progress**

- o Assess the pathological stage and grade by immunohistochemical techniques of the collected tissue samples with the assistance of Dr. Nancy Joste, Chief of Surgical Pathology.
- In year one, the pathological stage and grade were assessed on 12 of the initial 17 cases.
- In year two, the pathological stage and grade were assessed on the other 5 cases collected in year one and on 7 of the 11 cases collected in year two.

Task 3 Months 6-32 **In Progress**

- o Isolate genomic DNA from tumor and CHN tissue specimens and determine extent of AI as a function of distance from tumor margin.
- In year one, isolation of genomic DNA and determination of the extent of AI was determined in 12 of the 17 collected cases (Appendix A).
- In year two, isolation of genomic DNA and determination of the extent of AI was determined in the remaining 5 cases collected in year one and in 5 of the 11 cases collected in year two.

Task 4 Months 6-32 **In Progress**

- o Isolate stromal and epithelial cell populations from selected CHN tissue specimens by LCM.
- The candidate has completed training for use of the LCM machine.

Task 5 Months 6-32 **In Progress**

- o Extract RNA from isolated cell populations.
- In year two, RNA was extracted from "bulk" (i.e. no isolation of specific cellular populations) tissues.

Task 6 Months 9-32 **In Progress**

- o Measure extent of AI in epithelial and stromal cell populations.
- In year two, the extent of AI was determined in the "bulk" tissues.

Task 7 Months 12-32 **In Progress**

- o Perform expression analysis using stromal and epithelial cell RNA from CHN tissues by microarray hybridization. Determine molecular signatures as a function of distance from the visible tumor margins using cluster analysis.
- In year two, due to limitations in RNA isolation from normal breast tissues, microarray hybridization experiments where performed on "bulk" breast tissues

1cm from tumor margin (N=6), breast tissues 5cm from tumor margin (N=6), and breast tissues obtained from reduction mammoplasty (N=5), and compared to 10 pooled RNAs from normal breast tissues. We identified 90 transcripts that were consistently over expressed by at least 2 standard deviations from the mean relative to the normal control in at least 5 of the 6 TAHN-1 tissues. Twenty two of these transcripts represented known genes, all but one was over expressed in all 6 TAHN-1 tissues, and 11 of the 22 have a documented involvement in breast cancer progression (Appendix F). We are currently validating these results with QRT-PCR and immunohistochemistry (IHC). The IHC experiments will show which cellular populations (epithelial or stromal) are over expressing the protein of interest.

Specific Aim 3 (5 tasks)

Task 1 Months 1-12 **In Progress**

- Procure fresh mastectomy specimens from the University of New Mexico Cancer Research and Treatment Center (UNM-CRTC). These may be same samples collected in aim 2.
- In year one, 17 cases (tumor, 1cm, 5cm tissues) were collected.
- In year two, an additional 11 cases were prospectively collected.
- In year two, due to limitations in the number of prospectively collected specimens, a cohort of retrospectively collected breast specimens, consisting of 52 reduction mammoplasty samples, 76 histologically normal tumor adjacent tissues, 34 benign breast disease cases, and 779 breast tumors (Stage 0-IV) was collected.

Task 2 Months 6-18 **In Progress**

- Assess the pathological stage and grade by immunohistochemical techniques of the collected tissue samples with the assistance of Dr. Nancy Joste, Chief of Surgical Pathology.
- In year one, the pathological stage and grade were assessed on 12 of the initial 17 cases.
- In year two, the pathological stage and grade were assessed on the other 5 cases collected in year one and on 7 of the 11 cases collected in year two.

Task 3 Months 6-18 **In Progress**

- Isolate DNA from breast tumors and measure AI.
- In year one, isolation of genomic DNA and determination of the extent of AI was determined in 12 of the 17 collected cases (Appendix A).
- In year two, isolation of genomic DNA and determination of the extent of AI was determined in the remaining 5 cases collected in year one and in 5 of the 11 cases collected in year two.
- In year two, AI was determined in the retrospectively collected cohort of breast specimens, consisting of 52 reduction mammoplasty samples, 76 histologically normal tumor adjacent tissues, 34 benign breast disease cases, and 779 breast tumors (Stage 0-IV).

Task 4 Months 24-30 **In Progress**

- Analyze the correlation between the AI and pathological stage.
  - In year two, the correlation between AI and pathological stage was assessed. The extent of AI was statistically different between normal, disease-free tissue and cancerous tissue and therefore may be used in a diagnostic setting. However, AI failed to discriminate between different stages of breast tumors (Appendix G).

Task 5 Months 18-36 **In Progress**

- Prepare and submit manuscripts.
  - Five manuscripts have been published (Appendices A, B, D, H, J) and two additional manuscripts have been submitted and are currently under review (Appendices C, E).

Education and Training Milestones (6 tasks)

Task 1 Months 1-6 **Completed**

- Learn to recognize morphology and features of different types of breast cancer under the guidance of Dr. Nancy Joste, Chief of Surgical Pathology.

Task 2 Months 6-12 **Completed**

- Learn staining procedures and significance of histological markers commonly used in breast cancer under the guidance of Dr. Nancy Joste, Chief of Surgical Pathology.

Task 3 Months 1-24 **In Progress**

- Interact with oncologists (Dr. Aroop Mangalik) in the University of New Mexico Hospital to gain perspective on breast cancer research.

Task 4 Months 1-36 **In Progress**

- Attend tumor board meetings and monthly Cancer Research and Treatment Center meetings to gain understanding of current treatments for breast cancer and ongoing clinical trials.
  - Due to HIPAA regulations, the candidate is no longer allowed to attend tumor board meetings. However, the candidate is still attending specialized departmental and Cancer Center seminars and is an active participant in the Breast Cancer Working Group through the Cancer Center.

Task 5 Months 12-18 **Not Initiated**

- Attend the University of New Mexico School of Medicine Undergraduate Medical Education Curriculum Neoplasia block.
  - The candidate planned to attend the Neoplasia block during year two; however, due to a scheduling conflict the candidate was unable to attend and plans to attend this upcoming academic year.

Task 6 Months 12-36 **In Progress**

- Present ongoing work at local and national meetings.

- In year one, the candidate presented work at three national meetings, two poster presentations and an oral presentation, and was a co-author on another poster presentation.
- In year two, the candidate presented a poster at the 1st Biennial National IDeA Symposium of Biomedical Research Excellence (NISBRE) (Appendix H).

III. REPORTABLE OUTCOMES

Publications:

C.M. Heaphy, M.Bisoffi, C.A. Fordyce, C.M. Haaland, W.C. Hines, N.E. Joste and J.K. Griffith. Telomere DNA content and allelic imbalance demonstrate field cancerization in histologically normal tissue adjacent to breast tumors. International Journal of Cancer, 119:108-116, 2006. (Appendix A)

B.J. Candia, W.C. Hines, **C.M. Heaphy**, J.K. Griffith and R.A. Orlando. Protease Nexin-1 expression is altered in human breast cancer. Cancer Cell International, 6:16, 2006. (Appendix I)

C.A. Fordyce,* **C.M. Heaphy***, M. Bisoffi, J.L. Wyaco, N.E. Joste, A. Mangalik, K. Baumgartner, R. Baumgartner, W.C. Hunt and J.K. Griffith. Telomere Content Correlates with Stage and Prognosis in Breast Cancer. Breast Cancer Research and Treatment, 99:193-202, 2006. **Authors contributed equally to this study* (Appendix B)

M. Bisoffi, **C.M. Heaphy** and J.K. Griffith. Telomeres: Prognostic markers in solid tumors. International Journal of Cancer, 119:2255-2260, 2006. (Appendix G)

C.M. Heaphy, W.C. Hines, K.S. Butler, C.M. Haaland, G. Heywood, E.G. Fischer, M. Bisoffi and J.K. Griffith. Measurement of Genome-wide Allelic Imbalance in Human Tissue Using a Multiplex PCR System. Journal of Molecular Diagnostics, 9:266-271, 2007. (Appendix D)

Manuscripts (submitted):

C.M. Heaphy, K.B. Baumgartner, M. Bisoffi, R.N. Baumgartner and J.K. Griffith. Telomere DNA Content Predicts Breast Cancer-free Survival Intervals. Clinical Cancer Resarch, 2007. (Appendix C)

E.G. Treat*, **C.M. Heaphy***, L.W. Massie, M. Bisoffi, A.Y. Smith, M.S. Davis and J.K. Griffith. Telomere DNA Content in Prostate Biopsies Predicts Early Rise In Prostate Specific Antigen Following Radical Prostatectomy for Prostate Cancer. Journal of Urology, 2007. **Authors contributed equally to this study* (Appendix E)

Published Abstracts:

C.M. Heaphy, C.A. Fordyce, M. Bisoffi, J.L. Wyaco, N.E. Joste, A. Mangalik, K. Baumgartner, R. Baumgartner, W.C. Hunt and J.K. Griffith (2006) Telomere content correlates with stage and prognosis in invasive breast cancer. 1st Biennial National IDeA Symposium of Biomedical Research Excellence (NISBRE). Washington, D.C. (Appendix I)

IV. CONCLUSIONS

To date, all tasks; as outlined in the Statement of Work are on schedule. The tasks outlined in Specific Aim #1 have been completed (#1-3) or are proceeding on schedule (#4). To date, a total of 496 node negative breast tumors have been obtained, of which 452 have been successfully analyzed for the extent of allelic imbalance (AI). However, we are still trying to procure 30 additional specimens from patients with node negative breast cancer that have progressed to recurrent disease. The tasks outlined in Specific Aim #2 and Specific Aim #3 have been initiated and are proceeding on schedule. Since the initiation of this training grant two years ago, five manuscripts have been published and two additional manuscripts have been submitted and are currently under review. The Ph.D. candidate is progressing with all of his educational goals and will attend the Neoplasia block at the University of New Mexico School of Medicine this upcoming academic year.

Int. J. Cancer: **119,** 108–116 (2006)

Telomere DNA content and allelic imbalance demonstrate field cancerization in histologically normal tissue adjacent to breast tumors

Christopher M. Heaphy[1], Marco Bisoffi[1,2], Colleen A. Fordyce[1], Christina M. Haaland[1], William C. Hines[1], Nancy E. Joste[2,3] and Jeffrey K. Griffith[1,2]*

[1]*Department of Biochemistry and Molecular Biology, University of New Mexico School of Medicine, Albuquerque, NM, USA*
[2]*Cancer Research and Treatment Center, University of New Mexico School of Medicine, Albuquerque, NM, USA*
[3]*Department of Pathology, University of New Mexico School of Medicine, Albuquerque, NM, USA*

Cancer arises from an accumulation of mutations that promote the selection of cells with progressively malignant phenotypes. Previous studies have shown that genomic instability, a hallmark of cancer cells, is a driving force in this process. In the present study, two markers of genomic instability, telomere DNA content and allelic imbalance, were examined in two independent cohorts of mammary carcinomas. Altered telomeres and unbalanced allelic loci were present in both tumors and surrounding histologically normal tissues at distances at least 1 cm from the visible tumor margins. Although the extent of these genetic changes decreases as a function of the distance from the visible tumor margin, unbalanced loci are conserved between the surrounding tissues and the tumors, implying cellular clonal evolution. Our results are in agreement with the concepts of ''field cancerization'' and ''cancer field effect,'' concepts that were previously introduced to describe areas within tissues consisting of histologically normal, yet genetically aberrant, cells that represent fertile grounds for tumorigenesis. The finding that genomic instability occurs in fields of histologically normal tissues surrounding the tumor is of clinical importance, as it has implications for the definition of appropriate tumor margins and the assessment of recurrence risk factors in the context of breast-sparing surgery.

Key words: telomere loss; allelic imbalance; genomic instability; cancer field effect; breast cancer

Genomic instability is an important factor in the progression of human cancers.[1–4] One mechanism that underlies genomic instability is loss of telomere function.[5–7] Telomeres are nucleoprotein complexes located at the ends of eukaryotic chromosomes. Telomeres in human somatic cells are composed of 1,000 to 2,000 tandemly repeated copies of the hexanucleotide DNA sequence, TTAGGG.[8] Numerous telomere binding proteins are associated with these repeat regions and are important for telomere maintenance.[9,10] Telomeres stabilize chromosome ends and prevent them from being recognized by the cell as DNA double-strand breaks, thereby preventing degradation and recombination.[11] However, telomeres can be critically shortened, and thereby become dysfunctional, by several mechanisms, including incomplete replication of the lagging strand during DNA synthesis,[12] loss or alterations of telomere-binding proteins involved in telomere maintenance,[13] and oxidative stress leading to DNA damage.[14] Alternatively, telomere loss may be compensated for by recombination[15,16] or, as seen in the majority of human cancers, by the enzyme telomerase.[17,18]

Telomeres in tumors are frequently shorter than in the matched adjacent normal tissues, presumably reflecting their extensive replicative histories.[19–21] The cause-and-effect relation between dysfunctional telomeres and genomic instability implies that shortened telomeres are also associated with altered gene expression. The latter is a primary source of phenotypic variability, which in turn drives the development of cell clones displaying progressively malignant traits, such as the potential for invasion and metastasis.[22] In agreement with this sequence of events, we and others have shown that telomere length, or its surrogate, telomere DNA content (TC), predicts the course of disease in several different malignancies, including leukemias,[23] non-small cell lung cancers,[24] neuroblastomas,[25] prostatic adenocarcinomas,[26–28] and breast carcinomas.[29,30]

Recently, Meeker and colleagues observed that telomere length abnormalities are early and frequent events in the malignant transformation of several types of cancer, including breast.[27,31,32] In addition, telomere attrition and other measures of genomic instability, such as allelic imbalance (AI) and loss of heterozygosity, demonstrate that genomic instability occurs within atypical breast hyperplasias,[33–35] histologically normal tissue proximal to breast tumors,[36–42] and, in some instances, breast tissue from women with benign breast disease.[43] Loss of heterozygosity and AI have also been found in the stromal compartment of cancer-associated breast tissues.[41,44] In addition, our own recent results identified fields of telomerase-positive cells within histologically normal tissues adjacent to breast tumors that could represent areas of premalignant cell populations.[45] Similarly, we have recently reported on the occurrence of telomere attrition in histologically normal prostatic tissue proximal to prostate adenocarcinomas.[28] These data imply that there is a reservoir of genetically unstable cell clones within histologically normal breast and prostate tissues that may represent fertile ground for tumor development. The origin and extent of this reservoir are presently undefined. However, the existence of fields of genetically altered cells, appearing histologically normal and disease-free, is consistent with the hypothesis that genomic instability arises early in breast tumorigenesis.

The primary goal of the present study was to define the extent and spatial distribution of genomic instability in histologically normal tissues surrounding breast tumors. A secondary goal was to investigate the relationship between genetic alterations in tumors and matched tumor-adjacent histologically normal (TA-HN) tissues. Towards these ends, two independent, yet conceptually linked markers of genomic instability, TC and AI, were investigated in two independent cohorts of breast tumors and their matched TA-HN tissues. One cohort represented a controlled study with tumors and matched TA-HN tissues excised at sites 1 and 5 cm from the tumor margins. The second cohort consisted of archival tumor specimens and matched TA-HN tissues excised at unknown distances from the tumor margin. Our results show that breast tumors reflect the properties of the matched TA-HN breast tissues, including the conservation of unbalanced alleles. Furthermore, our results support the hypothesis that fields of histologically normal, but genetically unstable cells provide a fertile ground for tumorigenic events in breast tissues.

Materials and methods

Breast tissue samples

Four independent cohorts of human breast tissues were used in this study. The characteristics of each of these cohorts are sum-

***Correspondence to:** Department of Biochemistry and Molecular Biology, MSC08 4670, 1 University of New Mexico, Albuquerque, NM, 87131-0001, USA. Fax: +1-505-272-6587.
E-mail: jkgriffith@salud.unm.edu
C.A. Fordyce's current address is Department of Pathology, University of California at San Francisco, San Francisco, CA, USA.
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TABLE I – CLINICAL CHARACTERISTICS OF TUMOR COHORTS

Cohort	*N*	Age at Dx[1]			Dx[1]			Size[2]		Node[3]		TNM Stage						
		Range	Median	Mean	IDC	LC	DCIS	S	L	N	P	n/av	I	IIA	IIB	IIIA	IIIB	IV
1	12	26–61	53	49	10	1	1	n/av		2	10	2	0	3	2	2	3	0
2	38	35–75	48	50	36	2	0	4	32	7	29	2	2	5	14	11	0	2
3	48	31–89	54	56	44	4	0	8	40	19	29	0	11	13	15	8	1	0
4 (Normal)	20	15–48	30	29	n/a	n/a	n/a	n/a		n/a		n/a						

TNM, Tumor-Nodes-Distant Metastasis; n/a, not applicable; n/av, not available.
[1]Dx, Diagnosis of invasive ductal carcinoma (IDC), lobular carcinoma (LC), ductal carcinoma in situ (DCIS).–[2]S = small (≤2 cm), L = large (>2 cm).–[3]N = negative, P = positive.

marized in Table I. The first cohort consisted of 12 full mastectomy cases obtained consecutively from the University of New Mexico (UNM) Hospital Surgical Pathology Laboratory in 2003 and 2004. Approximately 500 mg of tissue was excised from the tumors and sites 1 and 5 cm from the visible tumor margins. After resection, the tissues were immediately frozen in liquid nitrogen. Sections (10–12 μm) were prepared and stained with hematoxylin and eosin by the Human Tissue Repository Service of the UNM Department of Pathology. The sections were examined microscopically to define their histological status. In addition, serial sections of the breast tumors were collected and stored at −70°C until used for isolation of genomic DNA.

The second cohort was provided by the New Mexico Tumor Registry (NMTR) and consisted of 38 archival, paraffin-embedded ductal or lobular carcinomas and matched, histologically normal breast tissues from women who had undergone radical mastectomies or lumpectomies between 1982 and 1993. The histologically normal breast tissues originated from different blocks than the tumor tissues and were obtained at the time of dissection from sites outside the visible tumor margins. Generally, the sections were selected to contain high epithelial cell fractions.

The third cohort was obtained from the University of New Mexico Solid Tumor Facility and consisted of 48 frozen archival invasive ductal or lobular carcinomas from women who had radical mastectomies or lumpectomies between 1982 and 1993. Unlike cohorts 1 and 2, matched, histologically normal breast tissues were not available for the tumors in cohort 3.

The fourth cohort was obtained from the National Cancer Institute Cooperative Human Tissue Network (Nashville, TN) and contained 20 normal, disease-free breast tissue samples from women undergoing reduction mammoplasty (NBRST-RM). In addition, peripheral blood lymphocytes (PBLs) were obtained from 59 women previously diagnosed with breast cancer. The women ranged in age from 25 to 74 years, with a mean of 53 years. All tissues used in this study were anonymous, and experiments were performed in accordance with all federal guidelines as approved by the University of New Mexico Health Science Center Human Research Review Committee.

TC assay

Telomere length measurements can be affected by both extraneous factors, such as tissue specimens' age and means of preservation and storage, and inherent properties, such as patients' ages and health status, and the organ sites from which the tissue specimens were collected. To minimize the confounding effects of extraneous factors, we previously described a slot blot method for titrating the TC in fresh, frozen or paraffin-embedded tissues up to 20 years old.[46,47] TC measured by this method is directly proportional to telomere length measured by Southern blot.[47] However, in contrast to Southern blotting, the TC assay can be performed with as little as 5 ng of genomic DNA,[46] and is insensitive to fragmentation of DNA to less than 1 kb in length.[47] Thus, there is excellent agreement between TC measured in paired tissues stored either frozen, or formalin-fixed in paraffin at room temperature.[28,30] Therefore, TC is a sensitive and convenient proxy for telomere length, particularly for applications where genomic DNA is fragmented or scant, such as in sections of archival, paraffin-embedded tissues comprising the second cohort of breast tumors, which contains specimens that are over 20 years old.

TC was measured as described previously.[46] Briefly, DNA was isolated from frozen or paraffin-embedded tissues and blood samples, using Qiagen DNeasy Tissue kits (Qiagen, Valencia, CA) and the manufacturer's protocols. DNA was denatured at 56°C in 0.05 M NaOH/1.5 M NaCl, neutralized in 0.5 M Tris/1.5 M NaCl, and applied and UV cross-linked to Tropilon-Plus blotting membranes (Applied Biosystems, Foster City, CA). A telomere-specific oligonucleotide, end-labeled with fluorescein, (5′-TTAGGG-3′)$_4$-FAM (IDT, Coralville, IA), was hybridized to the genomic DNA, and the membranes were washed to remove nonhybridizing oligonucleotides. Hybridized oligonucleotides were detected by using an alkaline phosphatase-conjugated anti-fluorescein antibody that produces light when incubated with the CDP®-Star substrate (Applied Biosystems, Foster City, CA). Blots were exposed to Hyperfilm® for 2–10 min (Amersham Pharmacia Biotech, Buckinghamshire, UK) and digitized by scanning. The intensity of the telomere hybridization signal was measured from the digitized images, using Nucleotech Gel Expert Software 4.0 (Nucleotech, San Mateo, CA). TC is expressed as a percentage of the average chemiluminescent signal of three replicate tumor DNAs compared to the same amount of a placental DNA standard (typically 20 ng). In addition to placental DNA, DNA purified from HeLa cells, which has approximately 30% of placental TC was frequently included to confirm the reproducibility of the assay.

AI assay

DNA (approximately 1 ng) was amplified using the AmpFlSTR Identifiler PCR Amplification Kit (Applied Biosystems, Foster City, CA), using the manufacturer's protocol. Each multiplex PCR reaction amplifies 16 short tandem repeat (STR) microsatellite loci from independent locations in the genome (Amelogenin, CSF1PO, D2S1338, D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51, D19S433, D21S11, FGA, TH01, TPOX and vWA). Each of the PCR primers is labeled with one of four fluorescent dyes (6-FAM, PET, VIC and NED), each with a unique emission profile, allowing the simultaneous resolution of 16 amplicons of similar size. PCR products were resolved by capillary gel electrophoresis and detected using an ABI Prism 377 DNA Sequencer (Perkin Elmer, Foster City, CA). The height of each fluorescence peak in the electropherograms was quantitated using the ABI Prism GeneScan and Genotype Analysis software (Applied Biosystems, Foster City, CA) and a ratio of the peak heights of each pair of heterozygous allelic amplicons was calculated. By convention, the allele with the greater fluorescence intensity was designated the numerator. Thus, the ratio was always ≥1.0, with 1.0 representing the theoretical ratio for normal alleles.

Statistical analysis

Statistical analyses were performed using the JMP® statistical package (SAS Institute, Cary, NC), choosing a significance level of 0.01. The nonparametric two-sided Wilcoxon/Kruskal–Wallis log rank test was used to determine the comparative distribution of TC and AI in the breast tumor and TA-HN tissue specimens, as well as associations between TC and AI in the paraffin-embedded breast tumor samples of cohort 2.

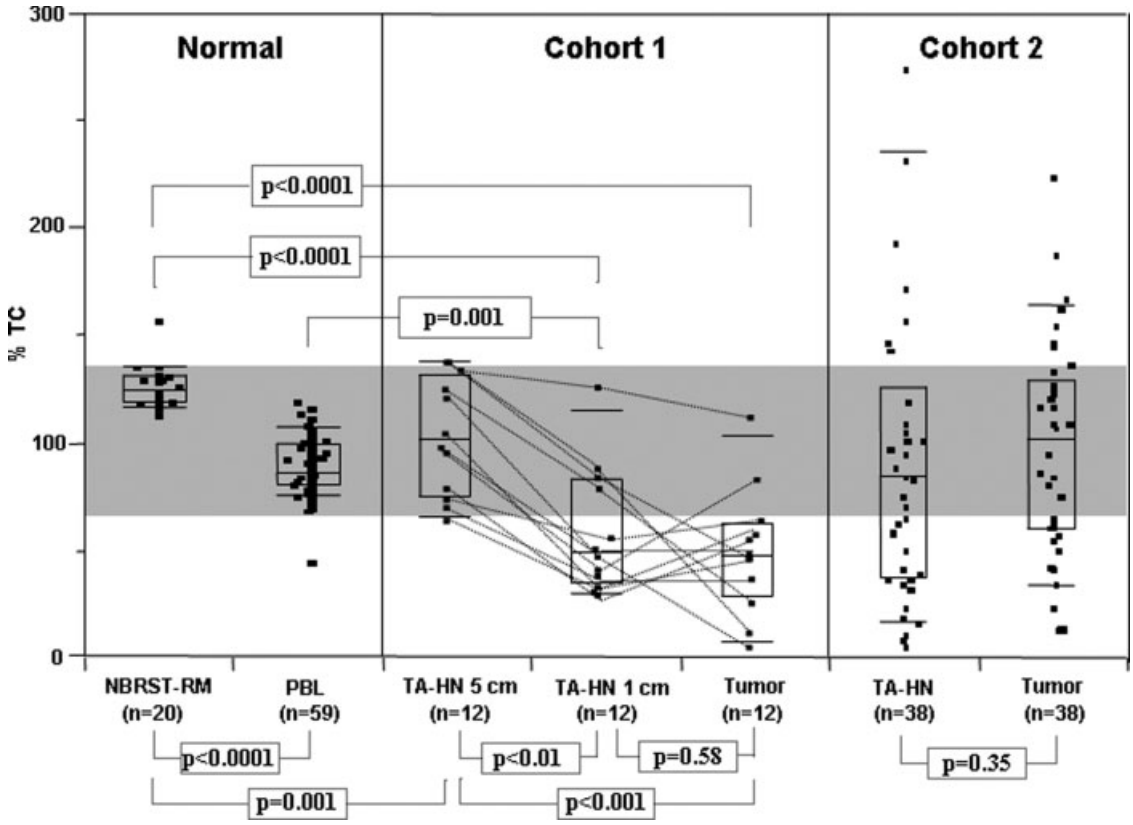

Figure 1 – Distribution of telomere DNA content (TC) in disease-free normal breast tissues from reduction mammoplasties (NBRST-RM), in peripheral blood lymphocytes (PBL), and in the breast tumor cohorts 1 and 2, including their tumor-adjacent histologically normal (TA-HN) tissues. TA-HN was excised at 1 and 5 cm from the tumor margin in cohort 1, and at unknown distances from the tumor margin in cohort 2. The number of tissues analyzed is indicated (*n*). TC is expressed as percentage of TC in placental control. The boxes represent group median (line across middle) and quartiles (25th and 75th percentiles) at its ends. Lines below and above boxes indicate 10th and 90th percentiles, respectively. In cohort 1, TC values of the individual matched samples are connected by thin lines. The gray shaded area indicates 95% of TC measurement for all normal specimens (NBRST-RM and PBLs). The *p*-values indicate comparisons between different tissue cohorts calculated by the two-sided Wilcoxon Kruskal/Wallis rank sums test. Additional statistical comparisons are mentioned in the text. *Note:* (i) Although the data points are horizontally shifted, some are still overlapping, and therefore not visible; (ii) due to the scale of the figure, two data points at values of 404% and 480% in the TA-HN set of cohort 2 are not shown.

Results

TC in normal breast tissues

To define the normal range of TC in disease-free breast tissues, the TC, a proxy for telomere length,[46,47] was measured in normal breast tissues obtained from 20 women (mean age 29) undergoing reduction mammoplasty (NBRST-RM). TC ranged from 114% to 158%, with a mean of 127% and a median of 126%, of TC in the placental DNA standard (Fig. 1). The interquartile variation (IQR), a statistical measure of the dispersion of the data, was only 12%, indicating little variation in telomere length in normal breast tissue. For comparison, TC was also measured in PBLs from 59 women (mean age 53) with a previous diagnosis of breast cancer. TC in PBLs ranged from 46% to 120%, with a mean of 90%, a median of 87% and an IQR of 19%, of the standard. The mean TC in normal breast was significantly higher than mean TC in PBLs ($p > 0.0001$). However, greater than 95% of all normal specimens (NBRST-RM and PBLs) had TC values within 70–137% of the standard. This range is interpreted to include the effects of all extraneous and inherent factors on observed TC in normal tissue, including age, tissue site, sample source and experimental variation.

Histology of cancerous and adjacent histologically normal breast tissues

The histologies of the tissues comprising two representative cases from the two independent cohorts of breast tumor tissues and matched tumor adjacent histologically normal (TA-HN) tissues are shown in Figure 2. The first cohort was composed of 12 sets of breast tumor tissues and TA-HN tissues excised 1 cm (TA-HN-1) and 5 cm (TA-HN-5) from the tumor margins. Frozen sections were stained with hematoxylin and eosin and examined microscopically. Sections of the tumors contained variable amounts of infiltrating carcinoma and ductal carcinoma *in situ* (Fig. 2*A* and 2*D*). In contrast, both TA-HN-1 and TA-HN-5 tissues had normal architecture, lobular units, ducts, and adipose tissue (Fig. 2*B*, 2*C* and 2*E*, 2*F*, respectively). Unlike the first cohort, which was composed of snap frozen tissues derived from contemporary mastectomies, the second was composed of paraffin-embedded archival tissues derived from women who had radical mastectomies or lumpectomies between 1982 and 1993. Fig. 2 shows two representative pairs of hematoxylin and eosin stained tumor (Fig. 2*G* and 2*I*) and TA-HN tissues (Fig. 2*H* and 2*J*). Infiltrating carcinoma can be seen in the tumors, while the TA-HN tissues show normal lobular architecture. Although tumor and TA-HN tissues comprising the second cohort came from different paraffin blocks, and the TA-HN tissues were obtained from sites outside the visible tumor margins, the exact distances between the sites of the TA-HN tissues and the tumors' margins are not known.

TC in tumor and adjacent histologically normal breast tissues

The spatial distribution of TC was examined in the 12 groups of breast tissues comprising the first cohort and compared with TC in the normal, disease-free breast tissues from radical mastectomy (Fig. 1). The mean TC values in the TA-HN-5 and TA-HN-1 tissues

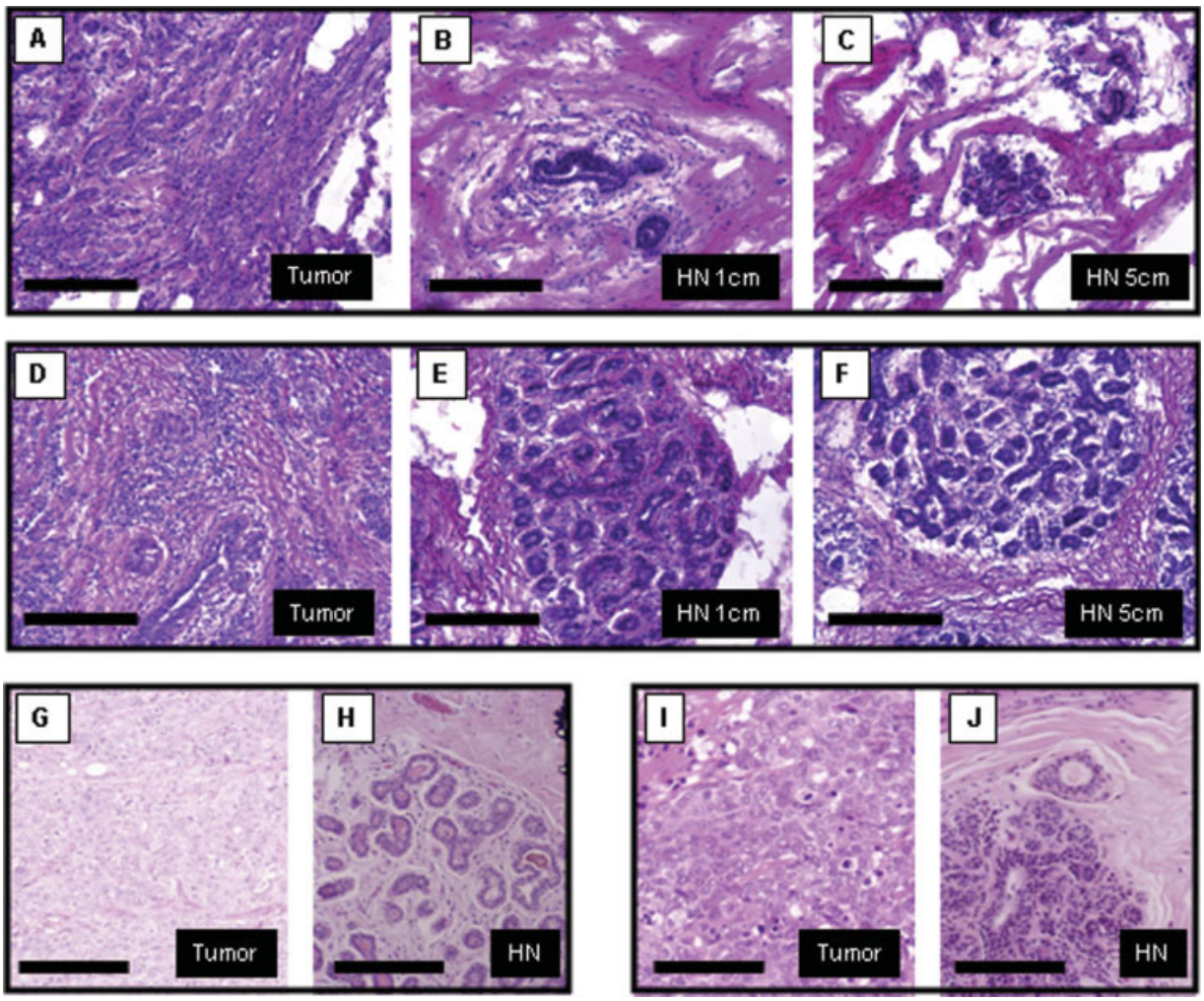

FIGURE 2 – Hematoxylin and eosin staining of human breast tissue sample sections. Two representative cases from the first (*A–F*) and second (*G–J*) cohorts are shown. Abnormal architecture with fields of infiltrating ductal carcinoma and ductal carcinoma *in situ* are seen in the tumor sections (A, D, G and I). Normal lobular and ductal architecture and adipose tissue are seen in the tumor-adjacent tissues at the indicated distance from the visible tumor margin (first cohort: B, C and E, F), or at unknown distances (second cohort: H and J). HN, histologically normal tissue; bars represent 200 μm.

were 101% and 66% of TC in the normal placental DNA standard, respectively. The mean TC value in tumors was 59%. Although the mean TC in TA-HN-5 tissues was significantly less than in NBRST-RM tissues ($p = 0.001$), it was not significantly different than the mean TC in PBLs from women of similar age ($p = 0.16$). Moreover, TC values in each of the TA-HN-5 tissues were within the range that defined >95% of all normal tissues. Since telomere length decreases with age,[48,49] it is likely that the difference between TC in the normal and TA-HN-5 tissues is due to the different ages of the two groups of women (27 *vs.* 49 years).

In contrast, mean TC in TA-HN-1 tissues was significantly less than TC in NBRST-RM tissues ($p < 0.0001$) and PBLs ($p = 0.001$), and TA-HN-5 tissues ($p < 0.01$). Mean TC in tumors also was significantly less than those in NBRST-RM tissues ($p < 0.0001$), PBLs ($p < 0.0001$) and TA-HN-5 tissues ($p < 0.001$). However, mean TC in tumor and TA-HN-1 tissues was indistinguishable ($p = 0.58$). Consistent with these findings, TC was, on average, 35% lower in each TA-HN-1 sample than in the paired TA-HN-5 sample, while the differences in TC between the TA-HN-1 and matched tumor specimens were varied, encompassing decrease, stabilization, and increase of TC with an average change of only 3% (lines in middle panel of Fig. 1). In total, TC values in 8 of 12 specimens of TA-HN-1 and 10 of 12 specimens of paired tumor tissues were outside the range that defined >95% of all normal tissues (NBRST-RM and PBLs).

Similarly, TC distribution was examined in a second, independent cohort (Fig. 1). Although the distributions of TC values in the 38 matched pairs of TA-HN and tumor tissues were broader than those measured in the first cohort (IQR = 88% and 69%, respectively), 16 of 38 TA-HN and 14 of 38 tumor specimens, respectively, had TC values less than those found in NBRST-RM tissues and PBLs, and only 9 of 38 TA-HN and 7 of 38 tumor specimens had TC values exceeding those found in all normal tissues (NBRST-RM and PBLs). A similar TC distribution was observed in a third collection of 48 frozen breast tumors (Table II), and in a collection of archival tumor and matched TA-HN prostate tissues, each collected between 1982 and 1993.[28] As observed in the comparison between tumor and TA-HN-1 specimens in the first cohort, there was no difference in mean TC in tumors and TA-HN tissues ($p = 0.35$). However, there was greater heterogeneity in the samples of the second as compared to the first cohort. Nevertheless, data from both cohorts are consistent with the conclusion that significant telomere attrition, comparable to that observed in tumors, occurs in TA-HN breast tissue. Significant telomere attrition (to a level outside the range seen in >95% of all normal tissues) occurred (i) in almost 50% (24/50) of TA-HN-1 and TA-HN

specimens, (ii) at sites at least 1 cm from the tumors' margins, and (iii) since TC is measured in bulk tissue that has not been microdissected, in a substantial fraction of the cells in the samples.

AI in tumor and adjacent histologically normal breast tissues

To investigate the extent of genomic instability in cohorts 1 and 2, tumor and TA-HN tissues were screened for AI at 16 unlinked microsatellite loci. Unlike the TC assay, which utilizes a slot blot methodology to titrate the quantity of telomere DNA in a defined amount of genomic DNA, the AI is defined by the ratio of the peak heights of allelic amplicons after PCR. Thus, it is unlikely that inherent or extrinsic factors that affect measurement of TC would similarly affect the determination of AI. To establish a baseline for the incidence of AI in normal breast tissue, 201 heterozygous loci in the 20 specimens of NBRST-RM tissues were analyzed by this approach. The mean peak height ratio was determined to be 1.18 (SD = 0.166). On the basis of these values, a highly conservative, operational definition of AI was established as a ratio of peak heights $\geq$1.68, *i.e.*, the mean + 3.0 SD. This threshold excluded more than 99% of the allelic ratios observed in the NBRST-RM tissues, and established a baseline incidence of 0.1 unbalanced loci per specimen of normal breast tissue. As shown in Figure 3, a virtually identical value, 0.08 loci per specimen, was measured in the TA-HN-5 tissues. In contrast, the mean numbers of unbalanced loci in the TA-HN-1 and tumor tissues were 0.42 and 1.25 loci per specimen, respectively, approximately 5 and 15 times higher than the incidence in the TA-HN-5 tissues. The baseline incidence of 0.1 unbalanced loci per specimen predicts that approximately 10% and 1% of normal tissues will have one and two unbalanced loci, respectively. Consistent with this prediction, 3 of 20 and 1 of 12 NBRST-RM and TA-HN-5 tissues, respectively, had one site of AI. Only one of more than 120 normal samples we have analyzed to date had 2 unbalanced loci, and none had more than 2 unbalanced loci. Accordingly, neither the NBRST-RM nor the TA-HN-5 specimens had more than one unbalanced locus. In contrast, one TA-HN-1, and 5 tumor tissues had 2 or more unbalanced loci. These data are consistent with the conclusion drawn from the TC analysis that both tumors and TA-HN-1 tissues are genetically distinct from TA-HN-5 tissue, and that both are genetically unstable.

TABLE II – TC VALUES IN NORMAL, TUMOR AND TUMOR ADJACENT, HISTOLOGICALLY NORMAL (TA-HN) TISSUES[1]

	N	Median	Mean	Min	Max	IQR
Normal tissues						
NBRST-RM	20	126	127	114	158	12
PBL	59	87	90	46	120	19
Cohort 1						
TA-HN-5	12	100	101	70	128	44
TA-HN-1	12	59	66	43	119	38
Tumor	12	57	59	24	108	27
Cohort 2						
TA-HN	38	85	106	6	480	88
Tumor	38	102	98	14	224	69
Cohort 3						
Tumor	48	105	118	65	247	60

IQR, interquartile range; NBRST-RM, normal breast tissue from reduction mammoplasty; PBL, peripheral blood lymphocytes.
[1]Data from Figure 1.

This conclusion is further supported by results obtained with the second cohort. Microsatellite alleles were successfully amplified in 23 pairs of the 38 samples. As with the TC determinations, the distribution of the numbers of unbalanced loci was much broader in the second cohort than in the first. The mean numbers of unbalanced loci in the TA-HN tissues and matched tumors were 2.61 and 2.48 loci per specimen, respectively (Fig. 3). The mean numbers of unbalanced loci in TA-HN and tumor tissues were significantly greater than the numbers in either NBRST-RM or TA-HN-5 tissues ($p < 0.01$). The extent of AI in the tumors and their matched TA-HN tissues of the second cohort were indistinguishable ($p = 0.88$). Significantly, 74% (17/23) of TA-HN tissues and 70% (16/23) of matched tumors had 2 or more sites of AI, and 57% (13/23) and 40% (9/23), respectively, had 3 or more sites. Like the TC measurements, the independent measurement of AI, performed in two independent cohorts of paired breast tissues, indicates that at least 1 unbalanced locus is present (i) in more than 74% (26/35) of TA-HN-1 and TA-HN specimens, (ii) at sites at least 1 cm from the tumors' margins and (iii) since AI was measured in bulk tissue that was not microdissected, and the threshold for detecting AI requires that approximately 40% of the cells have lost the specific allele (see later), specific sites of AI are present in a substantial fraction of the cells.

Conservation of unbalanced alleles in tumor and adjacent breast tissues

To investigate the possibility that TA-HN and tumor tissues represented early and late stages, respectively, in the clonal evolution of the cancers, we measured the frequency of conservation of unbalanced loci in the 2 cohorts of paired tumor and TA-HN tissues. As shown in Figure 4, in the first cohort, 2 of the 6 (33%) sites of AI present in TA-HN tissues were conserved in the paired tumors (left panel). Likewise, in the second cohort, 21 of the 60

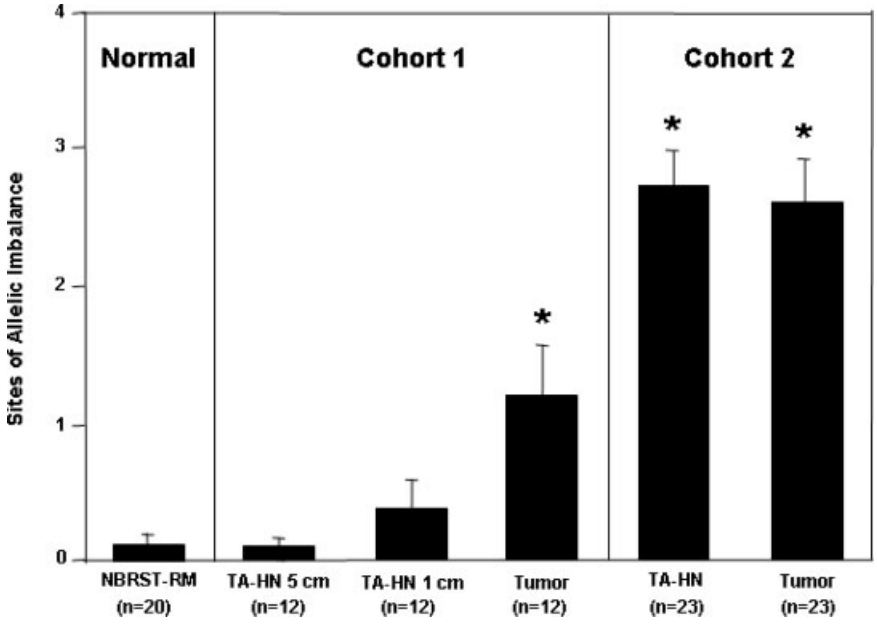

FIGURE 3 – Extent of allelic imbalance (AI) in disease-free normal breast tissues from reduction mammoplasties (NBRST-RM), and in the breast tumor cohorts 1 and 2, including their tumor-adjacent histologically normal (TA-HN) tissues. TA-HN was excised at 1 and 5 cm from tumor margin in cohort 1, and at unknown distances from the tumor margin in cohort 2. The number of tissues analyzed is indicated (*n*). The bars indicate the mean number of unbalanced loci ± standard errors. The stars indicate statistically significant differences ($p < 0.01$) from both NBRST-RM and TA-HN-5 (two-sided Wilcoxon Kruskal/Wallis rank sums test).

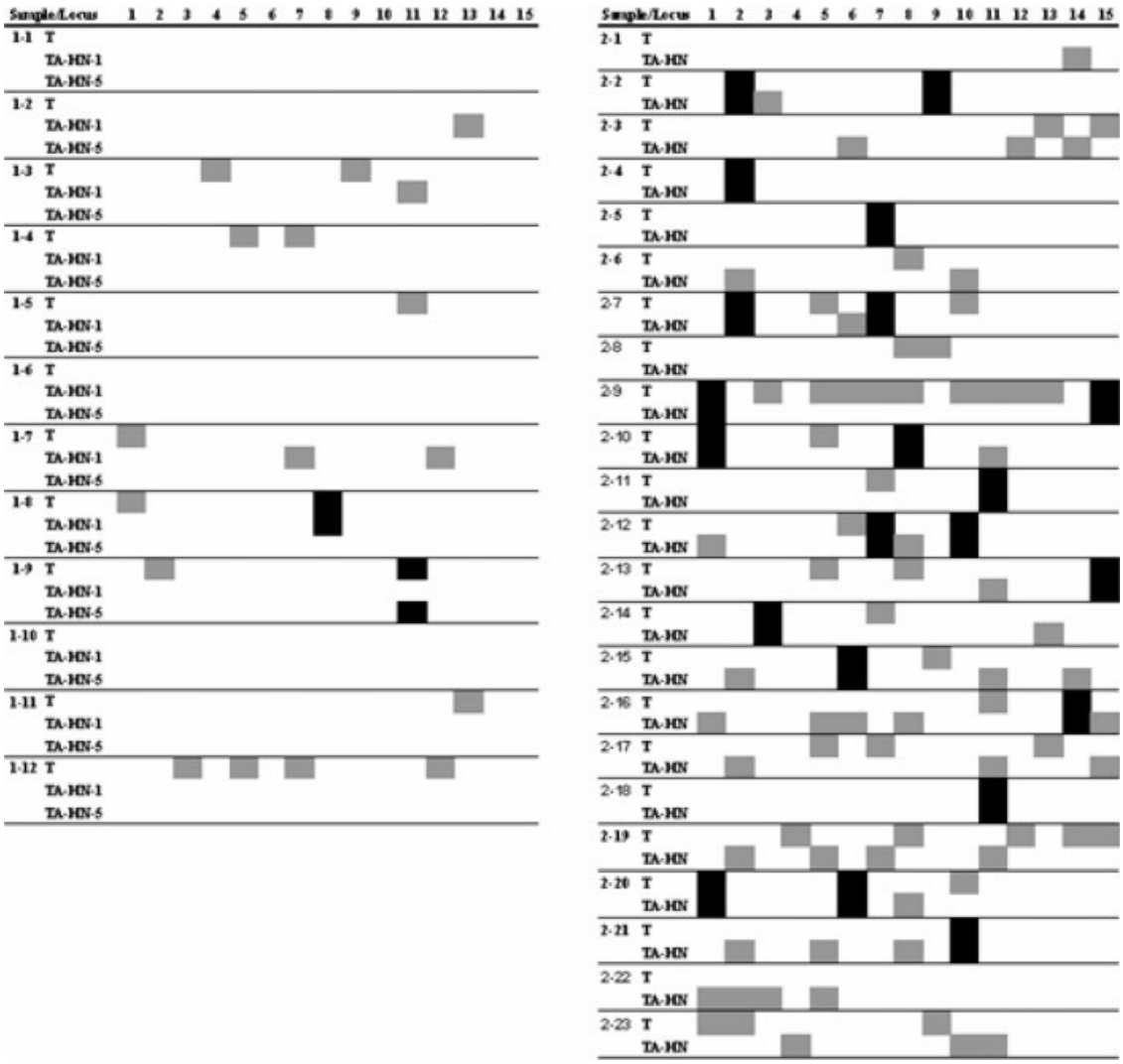

FIGURE 4 – Conservation of unbalanced alleles in matched tumor (T) and tumor-adjacent histologically normal (TA-HN) breast tissues of cohort 1 (left panel) and cohort 2 (right panel). Sites of allelic imbalances are indicated by gray boxes; sites of allelic imbalances conserved between tumor and TA-HN tissues are indicated by black boxes. The unlinked chromosomal loci are designated 1–15 and are as following (1) D8S1179, (2) D21S11, (3) D7S820, (4) CSF1PO, (5) D3S1358, (6) TH01, (7) D13S317, (8) D16S539, (9) D2S1338, (10) D19S433, (11) vWA, (12) TPOX, (13) D18S51, (14) D5S818, (15) FGA. *Note:* Homozygous amelogenin (all female samples) is not shown.

(35%) sites of AI present in TA-HN tissues were conserved in the paired tumors (right panel). The odds of this occurring by chance are estimated to be approximately 3×10^{-2} and 10^{-7} for the first and second cohorts, respectively.

Association between TC and AI in breast tumor tissues

Since telomere attrition is a source of genomic instability, and since we observed telomere attrition and increased AI in breast tumors, we determined the association between TC and AI (Fig. 5). For this analysis, microsatellite alleles were successfully amplified in 30 of the 38 breast tumor samples of cohort 2. Nonparametric 2-sided Wilcoxon/Kruskal–Wallis log rank analysis revealed a significant difference in TC in tumors with high ($\geq$3 sites) as compared to low ($\leq$2 sites) AI ($p = 0.002$).

Discussion

Although mechanistic insights into the molecular pathology of sporadic breast cancers are increasing, the question of how carcinogenesis is initiated in human breast tissues remains largely unanswered.[50–53] However, it is widely accepted that genomic instability is a prerequisite of virtually all tumors, including breast cancers, and that this instability facilitates the accumulation of further genetic alterations that result in cancer progression through clonal expansion of cells with a proliferative advantage.[1–3,51–53]

Two independent, quantitative measures of genomic instability, TC and AI, were used in this study to demonstrate that genomic instability occurs in histologically normal breast tissues adjacent to the corresponding tumors. These studies show that shortened telomeres (to a level outside the range seen in >95% of all normal tissues) and unbalanced allelic loci are present (i) in 50–75% of TA-HN and TA-HN-1 specimens, (ii) at sites at least 1 cm from the tumor margins and (iii) in a substantial fraction of the cells comprising the TA-HN tissue. This finding parallels our previous studies on tumors of the prostate and their matched TA-HN tissues,[28] and is in agreement with the work of previous investigators who reported that genetic alterations, including telomere attrition and loss of heterozygosity, occur in histologically normal tissues adjacent to breast tumors.[34–38,41–44] In these previous studies, the sites of telomere attrition, loss of heterozygosity and AI were physically distant from one another and from the tumors, albeit in most cases at undefined distances from the corresponding tumor lesions.[24,42–44] In contrast, and to our knowledge, the findings in cohort 1 represent the first

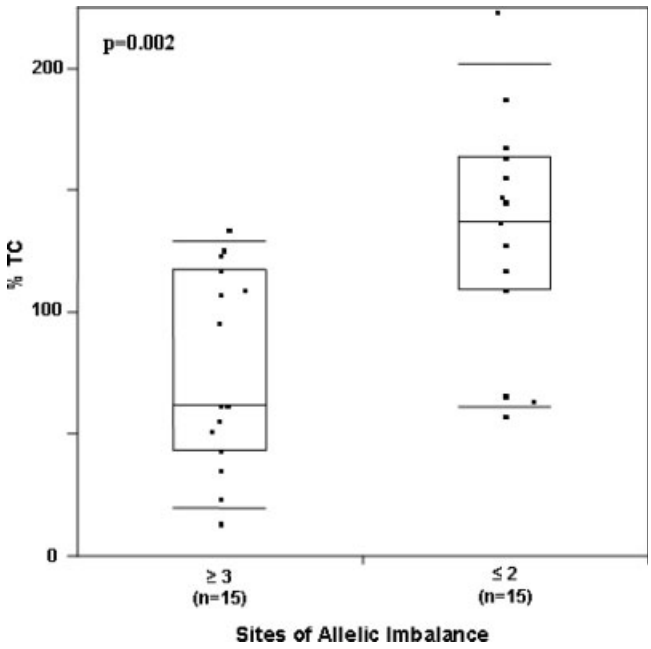

FIGURE 5 – Association between telomere DNA content and allelic imbalance in 30 breast tumor samples of cohort 2. The samples were dichotomized according to the number of genomic sites affected by allelic imbalance, i.e. ≥ 3 or ≤ 2 sites. The number of tissues analyzed is indicated (*n*). TC is expressed as percentage of TC in placental control. The boxes represent group median (line across middle) and quartiles (25th and 75th percentiles) at its ends. Lines below and above boxes indicate 10th and 90th percentiles, respectively. The nonparametric two-sided Wilcoxon/Kruskal–Wallis log rank test was used to assess the statistical significance of the difference between the means.

study in breast cancers that analyzes genomic instability at defined distances (1 and 5 cm) from the visible tumor margins. Consequently, this study reveals that genomic instability in tumor adjacent, histologically normal breast tissues is a function of distance from the tumor lesion, showing decreasing extent of genomic instability with increasing distance from the tumor margin. One explanation for these findings is that breast tumor cells exert a transforming effect on surrounding cells, leading to genetic alterations in adjacent tissues, as has been proposed for prostate cancer cells.[54,55] However, we prefer the alternate hypothesis, that breast epithelial carcinogenesis occurs at higher frequency in fields of cells with elevated genomic instability. This is supported by our observation that the occurrence of two independent markers of genomic instability, telomere attrition and unbalanced allelic loci, are highest in the tumor lesions and decrease with increasing distance from the tumor. In addition, analysis of tumors reveals an association between TC and extent of AI. Thus, we argue that telomere attrition induces genomic instability in breast tissues, and while this may not necessarily be apparent in histologically normal precancerous tissue, it is strongly displayed in tumor lesions.

Although similar conclusions can be drawn from the TC and AI analyses in each of the two cohorts, the range of TC values and the number of unbalanced loci per specimen were both greater in the second cohort. In this context, it is important to emphasize that both TC and AI reflect the average TC and peak height ratios in the cells comprising the sample; they do not provide information about the variability of TC or AI *between* individual cells. Consequently, the ability to detect specific changes in TC or AI diminishes as the number and types of cells in the sample increases. On the basis of the DNA yields, we estimate that there were approximately 20 times more cells in the samples comprising the first cohort (median $\sim 10^6$ cells), than the second cohort (median $\sim 5 \times 10^4$ cells). This difference reflects the relative amounts of tissue available from the fresh surgical specimens comprising the first cohort versus the sections of paraffin-embedded tissue blocks comprising the second cohort. This consideration is particularly significant in the case of the AI assay. On the basis of theoretical considerations and mixing experiments (data not shown), we estimate that imbalance at a specific locus must occur in $\sim$40% of the cells in the sample to generate an allelic ratio of 1.68, the threshold for significance used in these studies. Thus, sites of AI that are not prevalent in the cell population are not detected, even if there are many such individual sites. In this context, it is not surprising that specific sites of AI are detectable in breast tumors, which evolve clonally.[51] However, it is remarkable that AI is detected in TA-HN tissue, as it not only reflects underlying genomic instability, but also requires *clonal* expansion of genetically altered, premalignant cell clones within histologically normal breast tissues. This interpretation is further corroborated by the fact that more than a third of unbalanced alleles in adjacent, histologically normal tissues are conserved in the matched tumors. The latter has important practical implications, as it indicates that it is not necessary to micro-dissect tissues, for example using laser capture microscopy, to detect genomic instability, using the assays described in the present study. In fact, these assays allow the selective detection of changes in cell clones undergoing expansion because of proliferative advantages.

Taken together, our results are in agreement with the concept of ''field cancerization,'' introduced by Slaughter and colleagues in 1953,[56] and more recently reviewed by others.[57–59] These authors developed the term to explain the multifocal and seemingly independent areas of histologically precancerous alterations occurring in oral squamous cell carcinomas.[56] Organ systems in which field cancerization has been implied include lung, colon, cervix, bladder, skin and breast.[57] The concept of field cancerization has also been used to explain the occurrence of genetic and epigenetic mosaicism in cancer precursor tissues.[60] Based on our results, we propose to extend the concept of field cancerization to genetic alterations in otherwise histologically normal breast tissues, and our study is the first to include TC.

In head and neck squamous carcinoma, field cancerization has been shown for relatively large tissue areas, *i.e.* up to 7 cm in diameter.[61] It is thus not surprising that our data show extensive field cancerization in tissues 1 cm outside breast tumor margins. In the present study, TC was also different between disease-free NBRST-RM tissues and TA-HN tissues excised at 5 cm from the tumor margin. However, TC was similar in TA-HN-5 tissues and PBLs from women of similar age. Since telomere length decreases with age,[48,49] the observed difference in TC between NBRST-RM and TA-HN-5 tissues is likely due to the age discrepancy between the two cohorts of women (27 *vs*. 49 years).

The existence of fields of genomic instability that support tumorigenic events also has important clinical implications. First, such fields could give rise to clonal selection of precursor cells that ultimately lead to the development of cancer.[62] In this context, our recent studies have identified the presence of telomerase-positive cell populations within histologically normal tissues adjacent to breast tumors that could represent fields of premalignant cells.[45] Second, the presence of such fields, even after surgical resection of primary tumors, may represent an ongoing risk factor for cancer recurrence or formation of secondary lesions, which occurs in up to 22% of women undergoing breast conservation therapies for small invasive and noninvasive breast cancers.[58,63,64] For these reasons, our study has practical implications for the assessment of appropriate tumor margins for breast cancer surgical procedures, secondary treatment options and prognosis, possibly including the risk for the development of new primary tumors in the contra-lateral breast.[65–67] Thus, our study also suggests that evaluation of surgical margins should include molecular, in addition to histological, techniques, thus warranting further investigations.

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Preclinical study

Telomere content correlates with stage and prognosis in breast cancer

Colleen A. Fordyce[1,†], Christopher M. Heaphy[1,†], Marco Bisoffi[1], Jessica L. Wyaco[1], Nancy E. Joste[2], Aroop Mangalik[3], Kathy B. Baumgartner[4], Richard N. Baumgartner[3], William C. Hunt[4] and Jeffrey K. Griffith[1]

[1]*Department of Biochemistry and Molecular Biology, 1 University of New Mexico, Albuquerque, NM, USA;* [2]*Department of Pathology, 1 University of New Mexico, Albuquerque, NM, USA;* [3]*Department of Internal Medicine, 1 University of New Mexico, Albuquerque, NM, USA;* [4]*New Mexico Tumor Registry, University of New Mexico School of Medicine, 1 University of New Mexico, Albuquerque, NM, USA*

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Summary

Purpose. To evaluate the hypothesis that telomere DNA content (TC) in breast tumor tissue correlates with TNM staging and prognosis.

Experimental design. Slot blot assay was used to quantitate TC in 70 disease-free normal tissues from multiple organ sites, and two independent sets of breast tumors containing a total of 140 samples. Non-parametric Rank–Sums tests, logistic regression and Cox proportional hazards models were used to evaluate the relationships between TC and tumor size, nodal involvement, TNM stage, 5-year survival and disease-free interval.

Results. TC in 95% of normal tissues was 75–143% of that in the placental DNA standard, whereas only 50% of tumors had TC values in this range. TC was associated with tumor size ($p = 0.02$), nodal involvement ($p < 0.0001$), TNM stage ($p = 0.004$), 5-year overall survival ($p = 0.0001$) and 5-year disease-free survival ($p = 0.0004$). A multivariable Cox model was developed using age at diagnosis, TNM stage and TC as independent predictors of breast cancer-free survival. Relative to the high TC group (>123% of standard), low TC (<101% of standard) conferred an adjusted relative hazard of 4.43 (95% CI 1.4–13.6, $p = 0.009$). Receiver operating characteristic curves using thresholds defined by the TC distribution in normal tissues predicted 5-year breast cancer-free survival with 50% sensitivity and 95% specificity, and predicted death due to breast cancer with 75% sensitivity and 70% specificity.

Conclusions. TC in breast cancer tissue is an independent predictor of clinical outcome and survival interval, and may discriminate by stage.

Introduction

It is estimated that in the US in 2005 more than 200,000 women were diagnosed with breast cancer, and approximately 40,000 women died from this disease. Micrometastasis (metastatic cells that have escaped the primary tumor, but are currently undetectable) are a primary cause of breast cancer recurrence and mortality. Although TNM (Tumor size-Nodal involvement-Metastasis) is among the most informative of current prognostic markers for breast cancer [1–2], it often fails to discriminate between women who will have favorable and poor outcomes [1–5]. Thus, it is important to develop new markers that accurately predict the likelihood of breast cancer recurrence at the time of diagnosis.

Nearly a century ago, Boveri proposed that cancer resulted from altered genetic material. It is now widely accepted that genomic instability – the amplification, loss or structural rearrangement of a critical gene or genes – occurs in virtually all cancers [6]**.** The phenotype of a tumor is a reflection of its gene expression. Therefore, mechanisms that generate genomic instability, and thereby alter gene expression, play direct roles in tumor progression, including the development of aggressive tumor phenotypes like micrometastasis. Telomere dysfunction is one mechanism of generating genomic instability [7–9]. Telomeres are nucleoprotein complexes that protect the ends of eukaryotic chromosomes from degradation and recombination [10–12]. Due to incomplete replication, telomeres are shortened during each round of cellular replication [13]. Telomere shortening

[†]Colleen A. Fordyce and Christopher M. Heaphy contributed equally to this study.

may also be a consequence of double-strand DNA breaks, or changes in either the expression or function of any of the numerous proteins required for telomere maintenance [14–16]. Critically shortened, dysfunctional telomeres are prone to chromosome fusion and breakage [17], and in normal somatic cells lead to p53-dependent senescence and apoptosis [18]. However, these mechanisms are inactivated in cancer cells, for example, through p53 and Rb mutations. The direct relationship between dysfunctional telomeres, genomic instability and altered gene expression implies that tumors with the shortest telomeres have the most unstable genomes and, consequently the greatest probability of aberrant gene expression. Likewise, tumors with the longest telomeres would be expected to have fewer genomic alterations, and therefore, lower probability of containing cells with the phenotypes associated with disease recurrence. Accordingly, we and others have postulated that the mean telomeric DNA length in a tumor may provide a surrogate for phenotypic variability and therefore have prognostic potential in tumors [19–21].

There have been several investigations of the relationship between telomere length, or its proxies, and outcome in cancer. The most well characterized of these are in hematological cancers where it has been shown that telomere loss is associated with decreased survival in multiple types of leukemia and myeloma [22–24]. However, there have been few investigations of the prognostic potential of telomere length in solid tumors, which account for the majority of cancer incidence. Primarily, this is because the limited quantity and poor quality of DNA that is typically recovered from archival tissues precludes the use of Southern blotting techniques for the determination of telomere length.

To circumvent these problems, we previously described an alternative approach for measuring telomere length in genomic DNA obtained from fresh, frozen and, most importantly, paraffin-embedded tissues up to 20 years old [25,26]. The content of telomere DNA sequences (TC) in a DNA sample is titrated by hybridization with a telomere specific probe, and then normalized to the quantity of total genomic DNA in the same sample, thus controlling for the differences in DNA ploidy that are frequent in solid tumors. Our previous studies have shown that TC measured by this method is directly proportional to mean telomere length determined by Southern blotting [25]. Thus, TC is a proxy for telomere length and not affected by TTAGGG sequences outside the telomere. However, in contrast to Southern blotting, the TC assay can be performed with as little as 5 ng of genomic DNA and fragmented DNA less than 1 KB in length [25,26]. Therefore, the TC assay is particularly well-suited for analysis of retrospective studies of archival specimens from subjects with known outcomes.

Using this method, we previously demonstrated that reduced TC is associated with metastasis to lymph nodes in breast cancer [19]. More recently, we reported that TC was an independent predictor of time to prostate cancer recurrence (RH = 5.02) [20]. Short telomeres have also been associated with poor outcomes in neuroblastomas [27] while very long telomeres are a positive prognostic indicator in glioblastoma multiforme [28]. Collectively, these data imply that the extent of telomere loss or gain in tumors may have wide potential as a prognostic marker. However, this conclusion must be considered provisional, as prior studies often were based on small numbers of samples, highly selected patient populations and limited follow-up data using multiple clinical endpoints. In addition, the criteria for defining ''long'' or ''short'' telomeres are usually relative, and the relationships between telomere lengths in tumors and true disease-free tissue are often undefined.

In the current investigation, we have used the TC assay to define a normal range of telomere DNA content in breast and other tissues from multiple sites in healthy donors, compared this range to the distribution of TC measured in breast tumor tissues, and evaluated the relationships in breast tumor tissues between TC and TNM stage (and its individual components), 5-year breast cancer survival, and breast cancer-free survival interval following surgical excision of breast carcinoma.

Materials and methods

Tissue samples

Four independent sets of human breast tissues were used in this study. The first set (1982–1993) was comprised of 77 archival frozen and paraffin-embedded breast tumor tissues from women with either invasive ductal or lobular carcinomas who had radical mastectomies ($N = 63$), breast sparing surgery ($N = 11$) or unspecified surgeries ($N = 3$) between 1982 and 1993. The second set (1996–1999) was comprised of 63 archival paraffin-embedded breast tissues from a randomly selected subset of women participating in the population-based Health, Eating, Activity and Lifestyle (HEAL) Study [29]. These women were diagnosed with ductal carcinoma *in situ* (DCIS), invasive ductal carcinomas or invasive lobular carcinomas, and had radical mastectomies ($N = 11$) or breast sparing surgery ($N = 52$) between 1996 and 1999. Clinical data on breast tumors (Tables 1, 2) were ascertained by the New Mexico Tumor Registry (NMTR), a member of the Surveillance, Epidemiology, and End Results (SEER) Program of the National Cancer Institute. TNM stage was assigned using the 2002 revised criteria [30]. This study was approved by the University of New Mexico (UNM) Human Research Review Committee.

The third set was obtained from the National Cancer Institute Cooperative Human Tissue Network (Nashville, TN) and contained disease-free breast tissue from women who had reduction mammoplasty (RM). The fourth set included matched tumor and histologically normal breast (HNB) tissues collected at sites 5 cm from

Table 1. Characteristics of tumor tissues

Set[a]	*N*	Size (mm)					Node involvement			TNM stage						Number of deaths at 5-years of follow up	
		Median	Mean	Range	Q1	Q3	Yes (%)	No (%)	NA (%)	0 (%)	I (%)	IIA (%)	IIB (%)	IIIA (%)	IIIB (%)	IV (%)	NA (%)	Total (%)	Breast cancer related (%)[b]
1982–1993	77	30	34	8–80	20	49	51 (66)	25 (33)	1 (1)	0 (0)	11 (14)	20 (26)	25 (32)	16 (21)	1 (1)	2 (3)	2 (3)	27 (35)	17 (22)
1996–1999	63	14	16	0–65	7	20	15 (24)	41 (65)	7 (11)	11 (17)	32 (51)	12 (19)	6 (9)	1 (2)	0 (0)	0 (0)	1 (2)	9 (14)	0 (0)
Combined	140	20	26	0–80	12	34	66 (47)	66 (47)	8 (6)	11 (8)	43 (31)	32 (23)	31 (22)	17 (12)	1 (1)	2 (1)	3 (2)	36 (26)	17 (12)

Abbreviations: *N*, number of specimens; Q1, Q3, first and third quartile (the difference between Q1 and Q3 is the inter-quartile range, or IQR); NA, not available.

[a]Tissue sets are described in the Materials and methods section.

[b]Of the 10 subjects who died from causes other than breast cancer, six died from other cancers, and one each from dementia, hypertension, pulmonary embolism, and unknown causes.

the visible tumor margins from women receiving full mastectomies at UNM Hospital in 2003 and 2004. To determine the extent to which TC differed as a function of age, tissue of origin and disease-status, buccal cells (BUC) were obtained from healthy male and female college student volunteers and peripheral blood lymphocytes (PBL) were obtained from women previously diagnosed with breast cancer.

Histological review

Paraffin-embedded and frozen tissue sections were stained with hematoxylin and eosin and were examined microscopically. Tumor tissues typically contained from 75–100% tumor cells.

Determination of telomere DNA content (TC)

DNA was extracted from slides cut from frozen or paraffin-embedded tissue, and TC was measured as described [20,26]. Briefly, DNA was isolated from frozen or paraffin-embedded tissues, and blood samples using Qiagen DNeasy Tissue kits (Qiagen, Valencia, CA) and the manufacturer's protocols. DNA was denatured at 56 °C in 0.05 M NaOH/1.5 M NaCl, neutralized in 0.5 M Tris/1.5 M NaCl, and applied and UV cross-linked to Tropilon-Plus blotting membranes (Applied Biosystems, Foster City, CA). A telomere-specific oligonucleotide, end-labeled with fluorescein, (5′-TTAG GG-3′)$_4$-FAM, (IDT, Coralville, IA) was hybridized to the genomic DNA, and the membranes were washed to remove non-hybridizing oligonucleotides. Hybridized oligonucleotides were detected by using an alkaline phosphatase-conjugated anti-fluorescein antibody that produces light when incubated with the CDP-Star substrate (Applied Biosystems, Foster City, CA). Blots were exposed to Hyperfilm for 2–10 min (Amersham Pharmacia Biotech, Buckinghamshire, UK) and digitized by scanning. The intensity of the telomere hybridization signal was measured from the digitized images using Nucleotech Gel Expert Software 4.0 (Nucleotech, San Mateo, CA). TC is expressed as a percentage of the average chemiluminescent signal from three replicate determinations of each tumor DNA relative to the average chemiluminescent signal in the same amount (typically 20 ng) of a reference DNA standard (placental DNA). DNA purified from HeLa cells, which have approximately 30% of the TC in placental DNA, and samples prepared without DNA served as positive and negative controls, respectively.

Statistical methods

We compared the distribution of TC for normal and tumor specimens and, within tumor specimens, by tumor size, nodal involvement, and TNM stage using schematic plots and the non-parametric Rank–Sums (Kruskal–Wallis) test. Logistic regression was used to model the fraction of tumors <2 cm in size, node

Table 2. Ages at tissue collection and telomere DNA contents in normal and tumor tissues

Set[a]	*N*	Age at tissue collection					Telomere DNA content (% placental DNA control)				
		Median	Mean	Range	Q1	Q3	Median	Mean	Range	Q1	Q3
Normal											
RM	20	30	29	15–48	21	36	126	127	114–158	120	132
HNB	12	53	49	26–61	39	59	101	101	70–128	79	124
PBL	12	NA	NA	NA	NA	NA	87	91	71–117	78	106
Buccal	26	NA	NA	NA	NA	NA	110	114	89–148	100	126
Combined	70	36	36	15–61	25	51	116	112	70–158	98	126
Tumor											
HNB Matched	12	53	49	26–61	39	59	57	59	24–108	42	69
1982–1993	77	48	52	31–88	42	60	108	109	36–247	77	126
1996–1999	63	56	59	32–85	48	72	136	148	31–359	98	177
Combined	152	53	55	26–88	45	65	110	121	24–359	76	146

Additional details are found in the text and the legend to Figure 1. Abbreviations: N: Number of specimens, Q1, Q3: first and third quartile (The difference between Q1 and Q3 is the interquartile range, or IQR). NA: Not available.
[a]Tissue sets are described in the Materials and methods section.

negative status, and at each TNM stage as a function of TC. The results of the logistic regression models are shown as plots of predicted values against TC. We investigated the association between survival and TC using Kaplan–Meier survival plots for three categories of TC, which were based on tertiles of the TC distribution in normal specimens. Death from any cause and death due to breast cancer were evaluated separately in the survival analyses. Cox proportional hazards models were used to control for the confounding effects of TNM stage and age. SAS version 9.1 and JMP (SAS Institute) were used for all analyses. *P*-values < 0.05 were considered to be significant.

Results

Telomere contents in normal tissues

Telomere content can be affected by several inherent properties, such as patients' ages and health status, and the organ sites from which the tissue specimens were collected. To evaluate the potential variability in TC arising from inherent properties of tissues, TC was measured in a diverse sampling of 70 specimens of normal tissue from multiple organ sites (Figure 1). Specimens included breast tissue obtained by reduction mammoplasty (RM); histologically normal breast tissues excised from sites 5 cm from the breast tumor margins (HNB), buccal cells from healthy, young men and women (BUC) and PBL from women with a prior diagnosis of breast cancer (PBL). As summarized in Table 2, median TC in HNB and PBL sets (101 and 87%, respectively) were approximately 30% lower than median TC in the RM and buccal specimens (126 and 110%, respectively). Similarly, the median ages for the donors of the HNB set (53 years) was almost twice the median ages of the donors of the RM samples (30 years). Although the ages of the volunteers contributing the BUC and PBL samples were not collected, the BUC samples were obtained from college students in their early 20s, while the PBL samples were obtained from a subset of a larger study group with a median age of 58 years. Thus, the results are consistent with the accepted view that telomere length in humans decreases as a function of age [13].

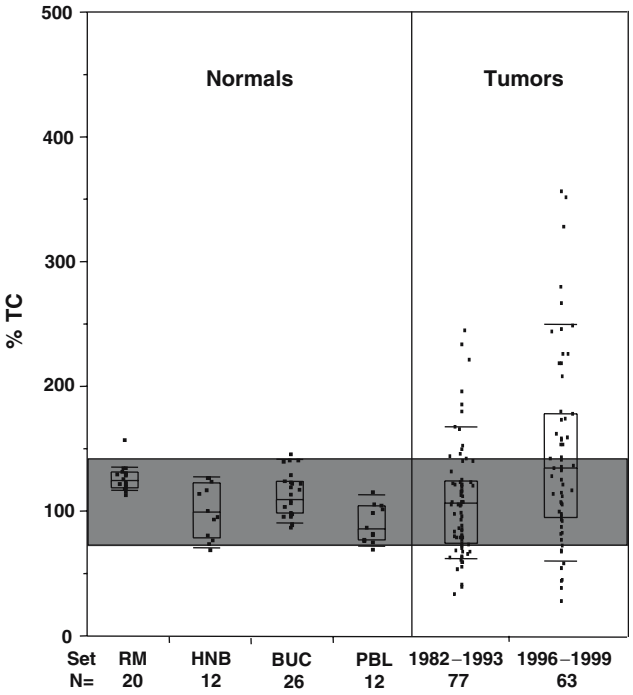

Figure 1. Distributions of telomere DNA contents (TC) in normal and tumor tissues. TC is shown on the *y*-axis, and is expressed as a percentage of TC in placental DNA standard, measured in parallel. The number of specimens in each tissue set (*N*) is indicated below the set designation on the *x*-axis. The shaded area (75–143% of the placental DNA standard) contains 95% of the TC values in the four sets of normal tissues. The line across the middle of each box shows the group median and the quartiles (25th and 75th percentiles) as its ends. The 10th and 90th quantiles are shown as lines above and below the box.

The inter-quartile range (IQR), a statistical measure of the dispersion of the TC data, was 28% for the combined normal tissues (Table 2). Ninety-five percent of all normal specimens had TC values of 75–143% of the standard (shaded area, Figure 1). In order to assess the extent to which this range was truly representative of normal tissues, we measured TC in a second, independent collection of 60 normal tissues (9 renal, 1 bone marrow, 2 breast, 2 lymph node, 2 prostate, 1 tonsil and 43 PBL). Similarly, 95% of the specimens had TC values within 75–145% of the standard (data not shown). Therefore, the distributions of TC in normal tissues is approximately 75–145%, which includes the effects of all extraneous factors, such as experimental variation, and inherent factors, such as subject's age and health status, the tissue type and source.

Telomere contents in breast tumor tissues differ from normal tissues

Matched tumor tissue was available for the 12 specimens of HNB tissues described above. Although TC in 11/12 of the HNB tissues fell within the expected range for normal tissues, only 2/12 matched tumors had TC within this range (Table 3). On average, TC in tumors was 61% of TC in the matched HNB tissues. TC was measured next in the 140 tumors comprising the 1982–1993 and 1996–1999 tumor sets (Figure 1). The IQR for TC in the two sets of tumor tissues, 49 and 79%, respectively, were substantially greater than the 28% IQR of the normal tissues (Table 2). Fifty-six percent of breast cancer specimens in the 1982–1993 set had TC values within the range that contained 95% of normal tissues, while 23 and 21% had TC values less and greater than the normal range, respectively. Similarly, only 43% of breast cancer specimens in the 1996–1999 set had TC values within the range that contained 95% of normal tissues, while 14% were below the range and 43% were above. Thus, TC in breast cancer tissues is significantly more heterogeneous than that in normal tissues, reflecting frequent abnormally short and long telomeres.

Table 3. TC in paired HNB and tumor tissue

Subject[a]	Telomere DNA content (% placental DNA control)		
	HNB (%)	Tumor (%)	T/N (%)
A	95	58	61
B	75	49	65
C	78	70	90
D	102	56	55
E	115	24	21
F	70	65	93
G	128	56	44
H	97	85	88
I	82	63	77
J	118	40	34
K	128	29	23
L	125	108	86
Average	101	59	61

Additional details are found in the text and the legend to Figure 1. T/N is the percent TC in the tumor (T) relative to TC in the paired, histologically normal (HNB) tissues.

[a]Tissue sets are described in the Materials and methods section.

Telomere contents in breast tumor tissues are associated with TNM stage

As shown in Table 2, mean and median TC differed between 1982–1993 and 1996–1999 tumor sets. A non-parametric Rank–Sums test of this difference in the means (109 and 148%, respectively) was highly significant ($p = 0.0008$). There were also highly significant differences between the two sets in the women's ages at diagnosis ($p = 0.001$), and their tumor's sizes ($p < 0.0001$), nodal involvements ($p = 0.0009$) and TNM stages ($p < 0.0001$). In order to more directly address a possible relationship between TC and the age at diagnosis, tumor size, nodal involvement and TNM stage, the two tumor sets were combined and these relationships were evaluated by non-parametric Rank–Sums tests (Figure 2a–c) and logistic regressions (Figure 2f–h). In each instance, there were highly significant associations with TC. Approximately 85% of the tumors in the 1982–1993 set were TNM stage IIA or higher; while approximately 66% of tumors in the 1996–1999 set were TNM stage 0 or I (Table 1). This, coupled with the strong association between TC and node status, suggests that TC discriminates across TNM stages. In contrast, there was no detectable association between TC and tumor histology (i.e. ductal versus lobular carcinomas).

Telomere contents in breast tumor tissues are associated with breast cancer survival

We hypothesized that telomere DNA length in a tumor is a surrogate for phenotypic variability and, therefore, atypically long and short telomeres, measured by high and low TC, respectively, are more likely associated with favorable and poor clinical outcomes, respectively. At least 5 years of follow-up data were available for 137 of the 140 women in the 1982–1993 and 1996–1999 sets. The relationships between TC and both overall 5-year survival and breast cancer-free 5-year survival were evaluated by non-parametric Rank–Sums tests (Figure 2d,e) and logistic regressions (Figure 2i,j). Both methods demonstrated highly significant associations between TC and overall 5-year survival ($p = 0.0001$, $p < 0.0001$, respectively) and breast cancer-free 5-year survival ($p = 0.0004$, $p = 0.0002$, respectively). The same conclusion was reached when the two tumor sets were analyzed separately (data not shown). In these analyses, the Kruskal–Wallis tests demonstrated that TC in the 1982–1993 group was associated with both overall 5-year survival ($p = 0.01$) and breast cancer-free 5-year survival ($p = 0.005$). TC in the 1996–1999 set was also associated with overall 5-year survival ($p = 0.02$)

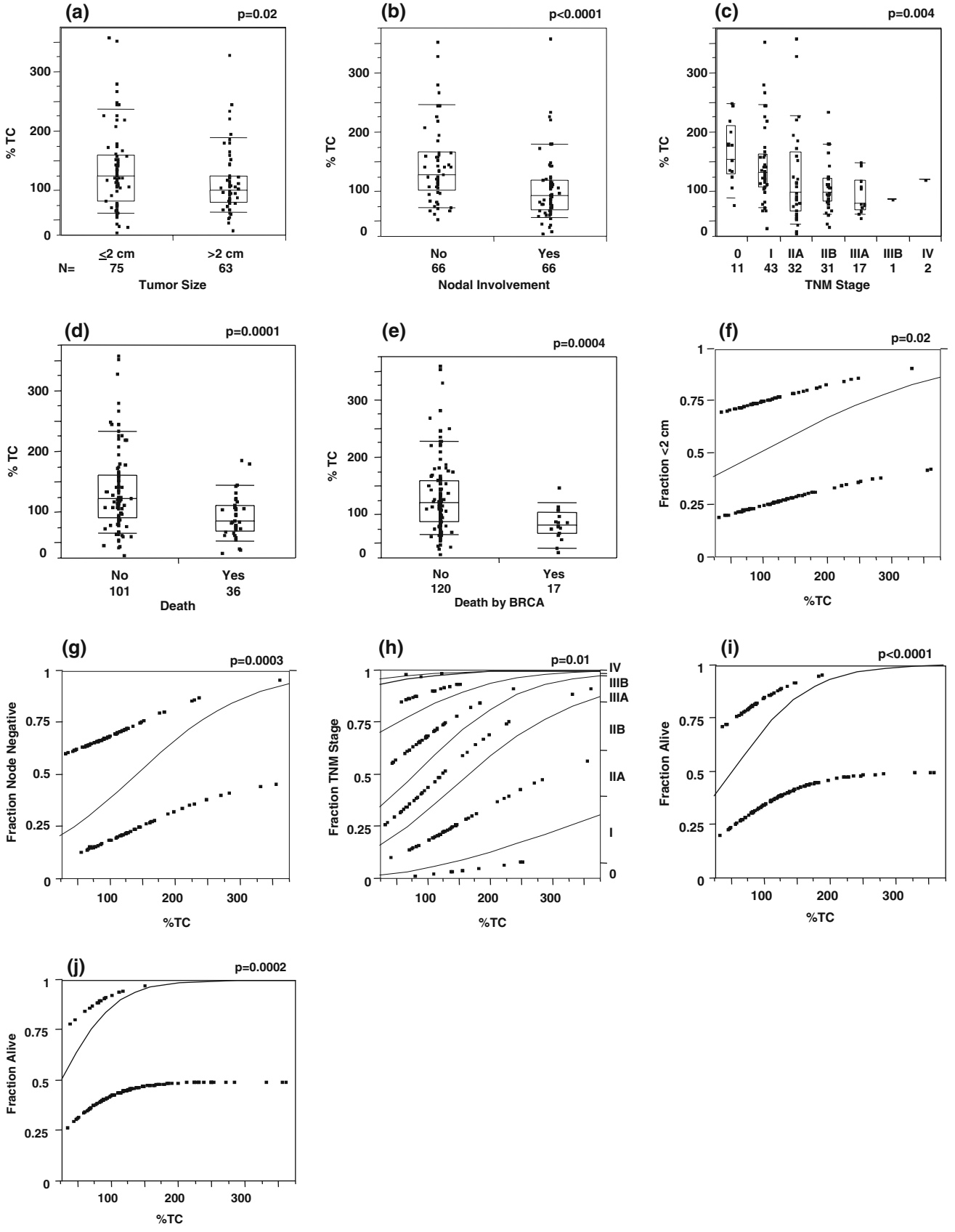
(a)
p=0.02
% TC
300
200
100
0
≤2 cm
>2 cm
N= 75 63
Tumor Size
(b)
p<0.0001
% TC
300
200
100
0
No
Yes
66 66
Nodal Involvement
(c)
p=0.004
% TC
300
200
100
0
0 I IIA IIB IIIA IIIB IV
11 43 32 31 17 1 2
TNM Stage
(d)
p=0.0001
% TC
300
200
100
0
No
Yes
101 36
Death
(e)
p=0.0004
% TC
300
200
100
0
No
Yes
120 17
Death by BRCA
(f)
p=0.02
Fraction <2 cm
1
0.75
0.5
0.25
0
100 200 300
%TC
(g)
p=0.0003
Fraction Node Negative
1
0.75
0.5
0.25
0
100 200 300
%TC
(h)
p=0.01
Fraction TNM Stage
1
0.75
0.5
0.25
0
IV
IIIB
IIIA
IIB
IIA
I
0
100 200 300
%TC
(i)
p<0.0001
Fraction Alive
1
0.75
0.5
0.25
0
100 200 300
%TC
(j)
p=0.0002
Fraction Alive
1
0.75
0.5
0.25
0
100 200 300
%TC

Figure 2. Associations between breast tumors' telomere DNA contents (TC) and tumor size, nodal status, TNM stage and 5 year breast cancer-free survival. Tumor sets 1982–1993 and 1996–1999 were combined and stratified by tumor size (a), nodal status (b), TNM stage (c), overall 5-year survival (d) and breast cancer-free 5-year survival (e). TC is shown on the *y*-axis, and is expressed as a percentage of TC in placental DNA standard, measured in parallel. The number of specimens in each tissue set (*N*) is indicated below the set designation on the *x*-axis. Statistical significance (*p*) was determined using the 2-sided non-parametric Rank–Sums test. The relationships between TC and tumor size (f), nodal status (g), TNM stage (h) overall 5-year survival (i) and breast cancer-free 5-year survival (j) were also evaluated by logistic regression. Logistic regression estimates the probability of choosing one of the specified parameters (e.g. large vs. small tumors) as a continuous function of TC. In a logistic probability plot, the *y*-axis represents probability. TC is shown on the *x*-axis, and is expressed as a percentage of TC in the placental DNA standard. The proportion of small tumors (i.e. <2.0 cm), node negative tumors, TNM stage 0–IV tumors, and survivors are shown on the *y*-axis. See the legend to Figure 1 for additional details.

however, no members of the 1996–1999 set died from breast cancer within 5 years of surgery (Table 1). Highly significant relationships between TC and overall 5-year survival ($p = 0.01$) and breast cancer-free 5-year survival ($p = 0.002$) in the 1982–1993 group, and overall 5-year survival in the 1996–1999 set ($p = 0.02$) were also detected by logistic regression. Collectively, the data support the conclusion that longer telomeres are protective while shorter telomeres presage poor survival.

The sensitivity and specificity of TC as a predictor of breast cancer-related death was evaluated by analysis of the TC's receiver operating characteristics (Figure 3). TC ranges for the lower, middle and upper tertiles in normal tissues were <101, 101–123, and >123% of standard, respectively. Consistent with the data in Figure 1 demonstrating that many tumors have TC values that are greater or lesser than those typically observed in normal tissues, only 20 and 14% of tumors in the 1982–1993 and 1996–1999 sets, respectively, had TC values within the range defined by the middle tertile. The 124% cutoff predicted 5 year survival with approximately 50% sensitivity and 95% specificity, while the 100% TC cutoff predicted death due to breast cancer with approximately 75% sensitivity and 70% specificity.

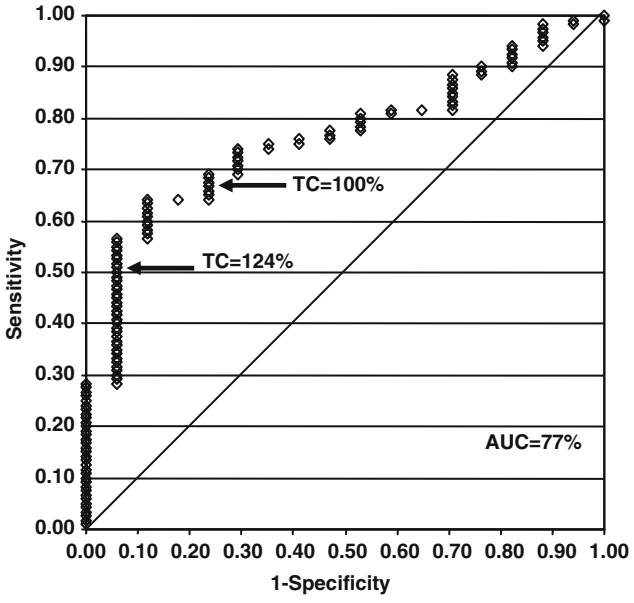

Figure 3. Receiver operating characteristics (ROC) analysis of the relationship between TC in breast tumors and 5 year survival. The specificity and sensitivity of TC as a predictor of 5 year survival following diagnosis of breast cancer was calculated for each value of TC in the combined 1982–1993 and 1996–1999 sets. Seventeen subjects died from breast cancer, and 119 survived for at least 5 years after diagnosis. The plot shows 1-specificity (the false positive rate) on the *x*-axis and sensitivity (1 – the false negative rate) on the *y*-axis. Arrows correspond to the high and low TC cutoffs (100 and 124%) that define the boundaries of the lower and upper tertiles of TC in normal tissues. See text for additional details. The area under the curve (AUC) is 77%.

Telomere contents in breast tumor tissues predict breast cancer-free survival interval

The extensive follow up data associated with the 77 tumors in 1982–1993 set (up to 23 years) made it possible to evaluate the effect of TC on breast cancer-free survival. The tumors were grouped using the TC thresholds described above: low TC was defined as less than or equal to 100%, intermediate TC was defined as 101–123%, and high TC was defined as greater than 123%. A Kaplan–Meier plot and Log–Rank test (Figure 4) demonstrated significant differences in the groups' survival intervals ($p = 0.013$). This effect is independent of age at diagnosis, nodal involvement and TNM stage (Table 4).

As shown in Table 5, low TC conferred an unadjusted relative hazard of 4.39 (95% CI = 1.47–13.08;

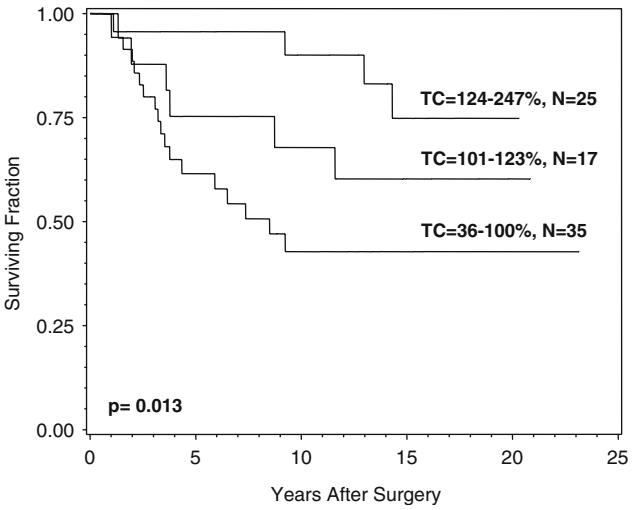

Figure 4. Breast cancer death by telomere DNA content in breast tumors. The 1982–1993 set was divided into three groups based on the high and low TC cutoffs (100 and 124%) that define the boundaries of the lower and upper tertiles of TC in normal tissues. Breast cancer-free survival interval, in years, is shown on the *x*-axis and the recurrence-free fraction is shown on the *y*-axis. The Log–Rank test was used to test the significance of the differences in the groups survival intervals ($p = 0.013$). *N* represents the number of subjects in each group. See Materials and methods section for additional details.

Table 4. TNM stage, lymph node involvement, mean age at diagnosis and tumor size by TC level

	TC level					
	36–100%		101–123%		124–247%	
	N	%	*N*	%	*N*	%
TNM stage						
I	3	8.6	3	17.6	5	20.0
IIA	11	31.4	2	11.8	7	28.0
IIB	12	34.3	6	35.3	7	28.0
IIIA, IIIB, IV	9	25.7	5	29.4	5	20.0
Unknown	0	0.0	1	5.9	1	4.0
Lymph nodes						
Negative	8	22.9	5	29.4	12	48.0
Positive	27	77.1	12	70.6	12	48.0
Unknown	0	0.0	0	0.0	1	4.0
	N	Mean	*N*	Mean	*N*	Mean
Age at diagnosis	35	56.3	17	46.9	25	48.5
Tumor Size (mm)[a]	35	36.1	15	32.1	25	32.1

Abbreviations: TC, telomere DNA content; *N*, number of specimens.
[a]Size is measured in longest dimension.

Table 5. Relative hazards and 95% confidence intervals from proportional hazards model of survival from date of diagnosis of breast cancer

	Unadjusted		Adjusted for age, TNM stage	
	RH (95% CI)	*p*-Value	RH (95% CI)	*p*-Value
TC level				
36–100	4.39 (1.47, 13.08)	0.0079	4.43 (1.44, 13.64)	0.0094
101–123	2.33 (0.66, 8.27)	0.1900	1.95 (1.54, 7.06)	0.3066
124–247	1.00		1.00	

A proportional hazards model of survival from date of diagnosis of breast cancer and up to 23 years of follow up was used to derive the unadjusted and adjusted relative hazards (RH) associated with each TC group. The adjusted RH was developed using age at diagnosis, TNM Stage and TC as independent predictors of survival. The 95% confidence intervals for RH are shown in parenthesis. Abbreviations: TC, telomere DNA content; RH, relative hazard; CI, confidence interval. See Materials and methods section for additional details.

$p = 0.008$) relative to high TC. A multivariable Cox model for the 1982–1993 breast tumor tissue set was developed using age at diagnosis, TNM stage and TC as independent predictors of breast cancer-free survival. Relative to the high TC group, low TC conferred an *adjusted* relative hazard of 4.43 (95% CI = 1.44–13.64; $p = 0.009$). In total these data demonstrate that TC predicts clinical outcome in invasive breast cancer.

Discussion

Telomere DNA content (TC) is a convenient proxy for telomere length that is particularly well-suited for the analysis of samples where DNA is degraded or scant, such as sections from archival, paraffin-embedded tissues. We measured TC values in three independent sets of cancerous breast tissues, compared these to TC in four sets of normal breast, buccal and blood cells, and evaluated the associations of TC with tumor markers and clinical endpoints, including disease-free and overall survival, in two independent cohorts comprising a total of 140 women with invasive breast cancer.

Four principal findings were made from this study. The first is that the range of telomere lengths in each of the three sets of breast tumors, measured as TC, is significantly greater than the range of TC in tissues from disease-free breast, buccal cells and blood cells. Only 17% of all tumors had TC values that were within the range defining the middle tertile of normal tissues, and approximately half of all tumors had TC values greater or lesser than those in 95% of normal tissues. These differences exceed those attributable to the several inherent and extraneous factors that can potentially confound measurements of telomere length, including age, and demonstrate the disparity between the regulation of telomere length in normal and tumor cells. It is significant that TC was associated with age in normal tissues, but not in tumors. This suggests that the extent of telomere attrition and the activities of the compensatory mechanisms that lengthen and stabilize telomeres, such as telomerase-dependant or -independent (“ALT”) processes, occurring in tumor cells are sufficiently large to obscure the underlying, age-dependent differences in telomere length.

Second, TC had significant associations with TNM stage (0 or I versus IIA and higher) and also two of its components: tumor size and nodal status. In contrast to previous studies, and our investigation of prostate tumors, where TC cutoffs were defined arbitrarily [20], TC cutoffs in the present study were derived from the distribution of TC values *in normal tissues*. Given the small amounts of DNA necessary to measure TC (as little as 5 ng), these results suggest that TC obtained by needle biopsy or fine needle aspirates (FNA) may be used to provide physicians preliminary TNM staging (or nodal involvement) information prior to surgery.

We next demonstrated an association between TC in breast tumor DNA and vital status following surgery. Even though the two tumor sets were not controlled for adjuvant therapies, the relationships between TC and overall 5-year survival and breast cancer-free 5-year survival were highly significant ($p = 0.0001$ and $p =$ c0.0004, respectively). TC thresholds based on the tertile distributions in normal tissues (described above) predicted 5 year breast cancer-free survival with approximately 50% sensitivity and 95% specificity and death resulting from breast cancer within 5 years of surgery with approximately 75% sensitivity and 70% specificity. Kaplan–Meier plots confirmed that TC was associated with the breast cancer-free interval.

Finally, TC provides prognostic information that is independent of its ability to discriminate disease stage. The relative hazard for death by breast cancer following diagnosis that is conferred by TC values $\leq 100\%$, after controlling for age at diagnosis and TNM stage involvement (RH = 4.43), was highly significant ($p =$ 0.009). This result is nearly identical to our prior finding that the relative hazard for recurrence of prostate cancer following prostatectomy conferred by TC values $\leq 75\%$, after controlling for age at diagnosis, Gleason sum, and pelvic node involvement (RH = 5.02) was also significant ($p = 0.013$) [20]. Together, these data support the hypothesis that TC provides *independent* prognostic information in multiple solid tumor types. We hypothesize that telomere content predicts the likelihood of micrometastasis and, in combination with extant prognostic markers, might have better predictive value than the extant markers alone, thus providing patients and their physicians new information to guide therapeutic decisions.

It is important to point out that all of the analyses reported herein were performed with DNA purified from tumor tissues that had *not* been microdissected. Although histological review of tissue sections indicated that tumor cells typically comprised 75–100% of the samples, the potentially confounding effects of contaminating normal cells in the tumor warrants consideration. In this context, we recently demonstrated that telomere attrition *comparable to that in matched prostate and breast tumor tissues* occurs in histologically normal tissues at distances at least one centimeter from the visible tumor margins [20,31]. In the latter study, it was estimated that at least 40% of the cells in the tumor adjacent histologically normal (TAHN) breast tissues were genetically aberrant, and more than a third of unbalanced alleles in the tumor were conserved in matched TAHN breast tissues, implying that the tumor and TAHN cells were derived from the same progenitor. Taken together, these data support the conclusion that TC in tumors and ''contaminating'' normal cells are comparable, thus precluding the requirement for tissue microdissection.

In summary, we report consistent differences in TC between normal, disease-free and cancerous breast tissues that are statistically significant by tumor characteristics and clinical outcome. We conclude that TC is a marker associated with disease stage and, importantly, appears to be an independent predictor of clinical outcome and survival.

Acknowledgments

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Address for offprints and correspondence: Jeffrey K. Griffith, Department of Biochemistry and Molecular Biology, University of New Mexico, 915 Camino de Salvel, Albuquerque, NM, 87131-0001, USA; *Tel.:* +1-505-272-3444; *Fax:* +1-505-272-6587; *E-mail:* jkgriffith@salud.unm.edu

Telomere DNA Content Predicts Overall and Breast Cancer-free Survival Intervals [#]

Christopher M. Heaphy[1], Kathy B. Baumgartner[3], Marco Bisoffi[1,2], Richard N. Baumgartner[3], and Jeffrey K. Griffith[1,2*]

Running Title: Prognostic Value of Telomere DNA Content in Breast Cancer

Keywords: Breast cancer, genomic instability, metastasis, prognosis, telomere

Affiliations: [1]Department of Biochemistry and Molecular Biology and the [2] Cancer Research and Treatment Center, University of New Mexico School of Medicine, Albuquerque, NM. [3]Current address: Department of Epidemiology and Population Health, School of Public Health and Information Science, University of Louisville, Louisville, KY.

[*]**Corresponding Author:** Jeffrey K. Griffith, Ph.D., Department of Biochemistry and Molecular Biology, MSC08 4670, 1 University of New Mexico, Albuquerque, New Mexico 87131-0001. Phone: 505-272-3444; Fax: 505-272-6587; Email: jkgriffith@salud.unm.edu

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ABSTRACT

BACKGROUND: Telomeres are nucleoprotein complexes that protect chromosome ends from degradation and recombination. Critically shortened telomeres generate genomic instability. It has been postulated that the extent of telomere DNA loss is related to the degree of genomic instability within a tumor, and therefore may presage clinical outcome. The objective of this investigation was to evaluate the hypothesis that telomere DNA content (TC) in breast tumor tissues predicts overall and breast cancer-free survival intervals.

METHODS: Slot blot titration assay was used to quantitate TC in 530 archival breast tumor tissues in a population-based cohort. The relationships between TC and twelve risk factors for breast cancer adverse events (i.e. death due to breast cancer, breast cancer recurrence, or new primary breast tumor) were evaluated by Fisher's Exact Test. The relationships between TC and survival intervals were evaluated by log-rank analysis and displayed by Kaplan-Meier survival plots. Multivariate Cox proportional hazards models were used to evaluate the relationships between TC and twelve risk factors for breast cancer-free survival interval.

RESULTS: A multivariate Cox proportional hazards model was developed using TC, p53 status, TNM stage, and ER status as independent predictors of breast cancer-free survival interval ($p<0.00005$). TC was independent of each of twelve risk factors. Low TC ($\leq$ 200% of standard), relative to the high TC group (> 200% of standard), conferred an adjusted relative hazard of 2.95 (95% CI=1.19-7.32; p=0.020) for breast cancer-related adverse events.

CONCLUSIONS: TC in breast cancer tissue is an independent predictor of overall and breast cancer-free survival intervals.

INTRODUCTION

Therapeutic management of breast cancer is complicated by the reality that conventional prognostic markers, such as patient age, TNM (Tumor size-Nodal involvement-Metastasis) stage and hormone receptor status, often do not identify women who will have a local or distant recurrence [1-3]. Hence, many women are unintentionally over- or under-treated. For example, approximately one-third of women with breast cancer are node-negative at the time of diagnosis, of whom about 80% and 70% will survive for 5 years and 10 years, respectively, if treated with surgery and radiotherapy alone [1]. Meta-analysis of approximately 30,000 breast cancer patients participating in 69 clinical trials indicates that adjuvant poly-chemotherapy in node-negative patients aged < 50 improves 10 year survival from 71% to 78%, while in patients aged 50-70, adjuvant therapy improves 10 year survival from 67% to only 69% [1]. However, because currently available staging and prognostic markers cannot reliably identify the minority of women who will benefit from adjuvant therapy, the NIH/NCI and St. Gallen guidelines each recommend adjuvant poly-chemotherapy for all women with moderate to high risk breast cancer [2,3]. Consequently, the majority of women with localized tumors have therapy-related side effects and reduced quality of life, while gaining no therapeutic benefit [4]. Thus, there is a pressing need for new markers that accurately predict the likelihood of breast cancer recurrence.

Tumorigenesis in humans is a multistep process in which successive genetic alterations, each conferring a selective advantage, drives the progressive transformation of normal cells into highly malignant cancer cells [5]. Due to incomplete replication, telomeres, the nucleoprotein complexes that protect the ends of eukaryotic chromosomes from degradation and recombination, are shortened during each round of cellular replication [6]. Most somatic cells do

not express telomerase, the reverse transcriptase responsible for adding the hexanucleotide sequence TTAGGG to the 3' strand of chromosomes, resulting in a reduction in telomere length with each cycle of chromosome replication [7,8]. Consequently, there is a limit to the number of doublings somatic cells can undergo before telomeres are critically shortened, become dysfunctional, and trigger an irreversible state of cellular senescence [9-11]. However, these protective mechanisms are inactivated in cancer cells, for example, through p53 and Rb mutations, resulting in successive rounds of chromosome breakage-bridge-fusion cycles, thus driving chromosome amplification, loss or structural rearrangement, and consequently, tumorigenesis [5,12].

The relationship between dysfunctional telomeres, genomic instability and altered gene expression implies that tumors with the shortest telomeres have the most unstable genomes and, consequently, the greatest probability of aberrant gene expression. Likewise, tumors with the longest telomeres would be expected to have fewer genomic alterations, and therefore, lower probability of containing cells with the phenotypes associated with disease recurrence. Accordingly, several recent studies suggest telomere length may provide independent prognostic information for several solid tumors, including breast cancers [reviewed in ref. 13].

Measurement of telomere length in formalin-fixed, paraffin-embedded (FFPE) tissues that are typically available for retrospective studies is problematic due to the limited quantity and poor quality of the DNA that is recovered. Methods that are not affected by these limitations, such as telomere fluorescent *in situ* hybridization (FISH), are not well suited for the high throughput analyses needed for large sample sets [14]. Moreover, because telomere lengths in the cells of

interest are defined by comparison to telomere lengths in reference cells in the same small field, there is potential for an increased sampling error.

To circumvent these problems, we previously described an alternative approach for measuring telomere length in genomic DNA obtained from fresh, frozen and, most importantly, FFPE tissues up to 20 years old [15,16]. The content of telomere DNA sequences (TC) in a DNA sample is titrated by hybridization on a slot blot with a telomere specific probe, and then normalized to the quantity of total genomic DNA in the same sample, thus controlling for the differences in DNA ploidy that are frequent in solid tumors. TC is particularly well-suited for use with DNA from archival tissues: TC is directly proportional to telomere length measured by Southern blot (r=0.904), can be measured with as little as 5 ng of genomic DNA, is insensitive to fragmentation of the DNA to less than 1KB in length, and can be measured successfully in DNA from FFPE tissues stored for up to 20 years at room temperature [15-18].

Using this method, we have recently demonstrated that TC is associated with breast cancer-free survival interval (RH=4.43; 95% CI 1.44-13.64; p=0.009), controlling for age at diagnosis and TNM stage [17]. This study and other investigations [reviewed in ref. 13] provide strong evidence that TC predicts clinical outcome. However, our previous study had a retrospective design (which is more open to bias than the current prospective study), included a limited number (N=77) of specimens collected in the mid 1980's and early 1990's, and was not controlled for the effects of adjuvant treatments and other clinical and prognostic parameters. Therefore, it is unknown how TC would perform as a prognostic marker in a contemporary,

population-based cohort, in which most tumors are detected by screening at earlier stages and many women elect breast sparing surgery with adjuvant radiation, chemo- or hormonal therapies.

In the current investigation, we addressed these questions by assessing the relationship between TC and both overall and breast cancer-free survival intervals in tumor specimens obtained from 530 members of the New Mexico subset of the NCI/SEER Health, Eating, Activity and Lifestyle (HEAL) prospective, population-based cohort [19].

MATERIALS AND METHODS

Tissue Samples: Five hundred and thirty breast tumor specimens were collected as a part of the prospective, population-based Health, Eating, Activity and Lifestyle (HEAL) Study [19]. The HEAL multi-center study was designed to evaluate the association between body composition, hormones, diet, physical activity, and prognosis over time for non-Hispanic white, Hispanic, and African-American women ascertained through the Surveillance, Epidemiology, and End Results (SEER) registries [http://appliedresearch.cancer.gov/surveys/heal/]. The eligibility criteria for the New Mexico (NM) arm of the HEAL study included a first primary breast cancer diagnosis, ascertainment through the New Mexico Tumor Registry (NMTR), age 18 or more years, residence in one of five centrally located NM counties, and diagnosis between July 1, 1996 and March 31, 1999. The TNM Stage of disease was based on the revised 2002 American Joint Committee on Cancer [20] stage groupings: 0 (*in situ*), I, IIA, IIB, and IIIA. Lymph node status, tumor size, age, chemo-, adjuvant and hormonal therapies, and menopausal status were based on medical record abstraction. Lymph node status was based on whether nodes were examined and the number identified as positive or negative for cancer. Ethnicity and family history were based on self-report at the time of interview. Coded data, stripped of all personal identifiers (Table 1), were provided by the HEAL investigators (RNB and KBB) and the NMTR, as approved by the University of New Mexico Human Research Review Committee. The mean age and follow up of cohort members were 59.1 (Range: 29-89; SD: 12.5) and 6.7 (Range: 0.45-9.16; SD: 1.6) years, respectively. At the time of analysis, 83% of the cohort members were alive. Additionally, 85% of the cohort members were free of disease, either at time of analysis or at time of their non-breast cancer related death.

Histological Review: FFPE tissue sections were stained with hematoxylin and eosin and were examined microscopically. Tissue sections were not microdissected and typically contained from 75-100% tumor cells.

Determination of Telomere DNA Content (TC): DNA was extracted from four 10μm FFPE tissue sections, and TC was measured by slot blot titration assay, as previously described [17,18]. Briefly, DNA was isolated using the Qiagen DNeasy Tissue kit (Qiagen, Valencia, CA) and following the manufacturer's protocol. DNA was quantitated fluorometrically with the dye, PicoGreen (Molecular BioProbes, Eugene, OR) [16], and known masses, typically 5-10 ng, were denatured at 56°C in 0.05 M NaOH/1.5 M NaCl, neutralized in 0.5 M Tris/1.5 M NaCl, and applied and UV cross-linked to Tropilon-Plus blotting membranes (Applied Biosystems, Foster City, CA). A telomere-specific oligonucleotide, end-labeled with fluorescein, $(5'\text{-TTAGGG-}3')_4$-FAM, (IDT, Coralville, IA) was hybridized to the genomic DNA, and the membranes were washed to remove non-hybridizing oligonucleotides. Hybridized oligonucleotides were detected by using an alkaline phosphatase-conjugated anti-fluorescein antibody that produces light when incubated with the CDP®-Star substrate (Applied Biosystems, Foster City, CA). Blots were exposed to Hyperfilm® for 5 min (Amersham Pharmacia Biotech, Buckinghamshire, UK) and digitized by scanning. The intensity of the telomere hybridization signal was measured from the digitized images using Nucleotech Gel Expert Software 4.0 (Nucleotech, San Mateo, CA). TC is expressed as a percentage of the TC in a placental DNA standard measured in parallel. Each measurement was repeated independently three times and the coefficient of variation for each sample was less than 10%.

Immunohistochemistry (IHC): IHC was performed on FFPE breast tumor sections to determine hormone receptor, p53 and HER2/*neu* status. Hormone receptor assays were conducted in laboratories associated with the hospitals where cases were diagnosed. p53 protein expression was evaluated using the anti-p53 monoclonal antibody, DO-7 (Santa Cruz Biotechnology, Santa Cruz, CA), which recognizes both the mutated and wild-type protein [21]. p53 tumor suppressor gene mutations occur in 20–50% of breast carcinomas [22], and have been reported to be associated with poor prognosis [23]. Mutations in p53 are predominantly mis-sense and lead to conformational alterations of the protein and accumulation in tumor cell nuclei. Consequently, immunohistochemical staining methods can be utilized for the detection of p53 protein as a simple substitute for mutational analyses [24-27]. HER2/*neu* protein expression was evaluated using the anti–HER2/*neu* monoclonal antibody, CB11 (Santa Cruz Biotechnology, Santa Cruz, CA).

Statistical Methods: The distribution of risk factors in the high and low TC groups (Table 1) was evaluated by the Fischer's Exact Test. Missing data for each risk factor was evaluated categorically in the analysis, but these data were not reported. The associations between TC and both overall survival interval and breast cancer-free survival interval were evaluated using log-rank Kaplan-Meier survival analyses. Multivariate Cox proportional hazards analysis was used to compute the relative hazards for breast cancer-related adverse events (i.e. death due to breast cancer, breast cancer recurrence, or new primary breast tumor). Covariate-adjusted estimates of the survival function by level of TC ($\leq 200\%$ *vs.* $> 200\%$) are the baseline survival estimates from a stratified proportional hazards model and were computed at the mean level of the

covariates. Subjects were censored at the time lost to follow-up. P-values less than 0.05 were considered significant for all tests.

RESULTS

Telomere Contents Predict Overall Survival: To confirm prior associations observed between TC and overall survival interval, the cohort was initially divided into sixths, the survival interval for each group was calculated, and the results were evaluated for statistical significance by log-rank analysis. Groups with statistically indistinguishable survival intervals were combined and the process was repeated until only groups with significantly different survival intervals remained. Using this process, the cohort was stratified into two TC groups: low TC was defined as $\leq 200\%$ in the placental DNA control (N=444), and high TC was defined as $> 200\%$ of TC in the placental DNA control (N=86), respectively. Log-rank analysis showed a significant relationship between TC group and overall survival interval (p=0.025), with low TC predicting a shorter survival interval. The results are plotted by the method of Kaplan and Meier and shown in Figure 1A. A univariate Cox proportional hazards model showed low TC had an unadjusted relative hazard of 2.25 (95% CI=1.09-4.64; p=0.029) relative to high TC (not shown). The relationship between TC group and overall survival interval in the subset of invasive tumors (i.e. without the 97 DCIS cases), was also evaluated. In this subset, log-rank analysis also showed a significant relationship between TC group and overall survival interval (p=0.046). The results are plotted by the method of Kaplan and Meier and shown in Figure 1B. A univariate Cox proportional hazards model showed low TC had an unadjusted relative hazard of 2.06 (95% CI=1.00-4.26; p=0.05) relative to high TC (not shown).

Telomere Contents Predict Breast Cancer-free Survival: Next, we refined our criteria to evaluate the prognostic value of TC in predicting breast cancer-related, adverse event-free survival interval. An adverse event was defined as death due to breast cancer, breast cancer

recurrence or development of a new primary breast tumor. Seventy-nine breast cancer-related adverse events had occurred by the time of the analysis, including 46 deaths, 15 recurrences and 18 new primary breast tumors. A Kaplan-Meier plot and log-rank test (Figure 2A) demonstrated significant differences in the groups' survival intervals (p=0.009), with low TC again predicting a shorter survival interval. A univariate Cox proportional hazards model showed low TC had an unadjusted relative hazard of 3.14 (95% CI=1.27-7.76; p=0.013) relative to high TC (Table 1). The relationship between TC group and breast cancer-free survival in the subset of invasive tumors was also evaluated. In this subset, log-rank analysis also showed a significant relationship between TC group and breast cancer-free survival interval (p=0.032). The results are plotted by the method of Kaplan and Meier and shown in Figure 2B. A univariate Cox proportional hazards model showed low TC had an unadjusted relative hazard of 2.61 (95% CI=1.05-6.48; p=0.039) relative to high TC (not shown). Similar, although not statistically significant, results were shown in the subset of DCIS cases (not shown).

Telomere Content is an Independent Predictor of Breast Cancer-free Survival: The relative hazards for breast cancer-related adverse events associated with twelve categorical risk factors were evaluated individually by Cox proportional hazards analysis (Table 1). Ethnicity, TNM stage, ER, PR and p53 status, chemotherapy sequence, adjuvant therapy, and TC each conferred significant ($p<0.05$) relative hazards. There was no significant hazard associated with age at diagnosis, family history of breast cancer, HER2/*neu* or post menopausal status or hormonal therapy. Analysis using Fisher's Exact Test showed no significant difference in the distribution of any of the risk factors in the low and high TC groups (Table 1).

Multivariate Cox proportional hazards models were developed using TC and other covariates either alone or in combination. The best overall model (Table 2) included TC, p53 and ER status, and TNM stage ($p<0.00005$). Relative to the high TC group, low TC conferred an *adjusted* relative hazard of 2.95 (95% CI=1.19-7.32; $p=0.020$). The chemotherapy, adjuvant therapy and hormonal therapy covariates were strongly associated with TNM Stage and each other ($p<0.0001$). Therefore, additional multivariate Cox proportional hazards models were developed using TC and chemotherapy, adjuvant therapy and hormonal therapy as covariates, either alone or in combinations. The best overall models included TC and either chemotherapy or adjuvant therapy ($p=0.002$); the addition of the hormonal therapy covariate had no effect. In the second model, low TC conferred an adjusted relative hazard of 2.84 (95% CI=1.14-7.05; $p=0.025$), relative to the high TC group (not shown).

DISCUSSION

Telomere DNA content (TC) is a convenient proxy for telomere length that is particularly well-suited for the analysis of samples where DNA is degraded or scant, such as sections from archival, FFPE tissues [15,16]. We used this method to determine TC values in tumor tissue collected in a prospective, population-based cohort comprised of 530 women, and evaluated the associations of TC with clinical parameters and endpoints, including overall and breast cancer-free survival intervals.

The principal conclusion from this investigation is that TC predicts both overall survival interval and breast cancer-free survival interval, independent of twelve clinical factors, prognostic markers and adjuvant therapies. Tumors with TC $\leq$ 200% of placental DNA standard conferred an *adjusted* hazard for breast cancer recurrence of 2.95 (95% CI 1.19-7.32; p=0.020). These results, obtained from a large population-based cohort, are in accord with our recent study [17] of breast tumors (predominantly TNM stage IIA and above) that also demonstrated highly significant associations between TC and overall 5-year survival ($p<0.0001$) and breast cancer-free survival interval (RH=4.43; 95% CI 1.44-13.64; p=0.009). Likewise, our previous investigation of prostate cancer [18] revealed that TC was also associated with time to prostate cancer recurrence (RH=5.02; 95% CI 1.40-17.96; p=0.013), controlling for age at diagnosis, Gleason sum, and pelvic node involvement. Similar results were obtained when analyses were performed using the subset of invasive tumors and a similar trend was observed in the subset of DCIS cases. These data suggest that TC may be able to predict clinical outcome in both invasive tumors and DCIS cases. As discussed above, adjuvant poly-chemotherapy in node-negative patients aged < 50 improves 10 year survival from 71% to 78% (a 24% increase, i.e. 7/29%),

while in patients aged 50-70, adjuvant therapy improves 10 year survival from 67% to only 69% (a 6% increase, i.e. 2/33%). A TC threshold of >200% of the standard defines a subgroup comprising approximately 17% of the population based cohort that have a significantly reduced risk of disease recurrence (7% at 8 years) that would be potential candidates for less aggressive adjuvant therapy. However, subsequent experiments in larger cohorts are needed to extend these findings.

The point estimate of the relative hazard for breast cancer recurrence associated with "low" TC was lower than in our prior investigation (2.95 *vs.* 4.43), although the confidence intervals overlap. One possibility is that the discrepancy in the point estimates reflects the difference in the length of follow-up in the two studies. The mean, maximum and interquartile range for follow-up in the HEAL cohort were 6.7, 9.2 and 1.5 years, respectively, versus 9.1, 23 and 11.2 years, respectively, in the prior study [17]. The ongoing follow-up of the HEAL cohort will resolve this question. It is also important to consider that HEAL is a prospective study in which FFPE tissue samples were collected for participants at multiple independent sites at the time of diagnosis before the start of follow-up, rather than a retrospective study of archival tissues from a single facility, which is more open to inadvertent selection bias.

Another important difference between these two studies is the TC threshold used to discriminate women at risk for breast cancer recurrence, > 123% and > 200% in the prior and present studies, respectively. This difference may also reflect the differences in the lengths of follow-up, in which case we would expect that the threshold will decrease as more deaths and adverse effects occur. Alternatively, the discrepancies in threshold, as well as the point estimates for the relative

hazard ratios, could reflect either the larger number of specimens (530 *vs.* 77), or the larger fraction of localized tumors (Stage 0 and I) in the HEAL cohort and prior cohort (67% *vs.* 14%).

Here, using the HEAL cohort, we have shown that TC predicts breast cancer-free survival interval, independent of other risk factors. It is important to note that these other established risk factors such as ethnicity, TNM Stage, ER, PR and p53 status, and chemo- and adjuvant therapy also conferred significant univariate relative hazards for breast cancer-related adverse events, confirming a representative population cohort. However, this population was not selected for TC (or any other biomarker) analysis, and thus represents an unbiased assessment of TC as a prognostic factor. Additionally, it must be noted that the cut-off established in this study, >200% of the placental DNA standard, exceeds the 95% CI for TC in several normal tissues (75-143% of standard), including breast [17]. Speculatively, these longer telomeres may result from the early up-regulation of telomerase during tumor progression.

In summary, TC in tissues from breast tumors is an independent predictor of overall and breast cancer-free survival intervals. In the future, TC in combination with extant prognostic markers could provide women and their physician's new information to guide therapeutic decisions. However, the assay in its current format, due to the relatively complex experimental procedure, is more suitable for use in a research rather than clinical setting. Therefore, development of a platform for TC determination that is simple and readily adaptable to a clinical laboratory is necessary before these findings can be validated in independent laboratories with independent cohorts.

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FIGURE LEGENDS

Figure 1. Overall Survival Interval by Telomere DNA Content in Breast Tumors. The set of all tumors (A) or invasive tumors only (B) was divided into two groups based on the low and high TC cutoff (200% of standard). Overall survival interval, in years, is shown on the x-axis and the surviving fraction is shown on the y-axis. Subjects were censored at the time lost to follow-up. The log-rank test was used to test the significance (p) of the differences in the group's survival intervals. N represents the number of subjects in each group.

Figure 2. Breast Cancer-free Survival Interval by Telomere DNA Content in Breast Tumors. The set of all tumors (A) or invasive tumors only (B) was divided into two groups based on the low and high TC cutoff (200% of standard). Breast cancer-free survival interval, in years, is shown on the x-axis and the recurrence-free fraction is shown on the y-axis. See Figure 1 for additional details.

Table 1. Relative hazards of risk factors for breast cancer-related adverse events in the HEAL patient cohort by TC level.

Characteristic	N	% of all patients (N=530)	RH (95% CI)	p	N	% of high TC (N=86)	N	% of low TC (N=444)
Ethnicity								
NHW	408	77	1.0		69	80	338	76
Hispanic	122	23	1.78 (1.11-2.84)	0.017	17	20	106	24
TNM Stage								
0 (*in situ*)	97	18	1.0		17	20	80	18
I	259	49	0.92 (0.46-1.86)	0.820	43	50	216	49
IIA	115	22	1.87 (0.91-3.85)	0.087	19	22	96	22
IIB	41	8	3.73 (1.71-8.13)	0.001	5	6	36	8
IIIA	5	1	1.94 (0.25-15.02)	0.527	0	0	5	1
ER Status								
Positive	444	84	1.0		71	83	373	84
Negative	82	15	2.62 (1.62-4.24)	0.0001	15	17	67	15
PR Status								
Positive	359	68	1.0		62	72	297	69
Negative	168	32	2.04 (1.31-3.18)	0.002	23	28	144	32
p53 Status								
Negative	262	49	1.0		44	51	218	49
Focal	151	28	0.99 (0.56-1.73)	0.966	19	22	132	30
Low	28	5	1.04 (0.37-2.94)	0.938	8	9	20	5
High	71	13	2.48 (1.44-4.27)	0.001	12	14	59	13
Age @ Dx								
$\leq$ 55	232	44	1.0		43	50	189	43
> 55	298	56	0.76 (0.49-1.18)	0.220	43	50	254	57
Family History								
None	244	46	1.0		41	48	203	46
1^0 Relative	128	24	0.87 (0.49-1.54)	0.627	20	23	108	24
2^0 Relative	108	20	1.05 (0.59-1.86)	0.873	17	20	91	20
HER2/*neu* Status								
Negative	300	57	1.0		49	57	251	57
Focal	111	21	0.95 (0.54-1.67)	0.845	16	19	95	21
Low	63	12	0.81 (0.38-1.71)	0.576	10	13	53	12
High	50	9	1.19 (0.58-2.44)	0.629	9	10	41	9
Chemotherapy								
None	406	77	1.0		71	83	335	75
After Surgery	118	22	1.91 (1.20-3.05)	0.007	15	17	103	23

Adjuvant Therapy								
None	178	33	1.0		26	30	152	34
Radiation	220	42	1.11 (0.63-1.98)	0.713	44	51	176	40
Chemo	30	6	3.25 (1.52-6.95)	0.002	1	1	29	7
Both	102	19	1.83 (0.99-3.38)	0.052	15	17	87	20
Tamoxifen								
Yes	250	47	1.0		45	52	205	46
No	280	53	0.71 (0.45- 1.12)	0.143	41	48	239	54
Post Menopausal								
No	156	29	1.0		25	29	131	30
Yes	358	68	0.79 (0.49-1.26)	0.323	59	69	299	67
TC								
> 200%	86	16	1.0		86	100	0	0
≤ 200%	444	84	3.14 (1.27-7.76)	0.013	0	0	444	100

TNM stage was assigned using the 2002 revised criteria [20]. Ethnicity and family history were based on self-report. Abbreviations: TC: Telomere DNA content, N: Number of specimens, ER: Estrogen Receptor, PR: Progesterone Receptor, NHW: Non-Hispanic White. See Materials and Methods Section for additional details.

Table 2. Relative hazards and 95% confidence intervals from a multivariate Cox proportional hazards model of breast cancer-free survival interval from date of diagnosis of breast cancer.

Variable	N	Level	RH (95% CI)	p value
TC				
	86	> 200%	1.00	
	444	≤ 200%	2.95 (1.19-7.32)	0.020
p53				
	262	None	1.00	
	151	Focal	1.01 (0.57-1.79)	0.977
	28	Low	1.27 (0.45-3.62)	0.652
	71	High	2.02 (1.11-3.67)	0.021
ER				
	444	Positive	1.00	
	82	Negative	1.67 (0.94-2.95)	0.079
TNM				
	97	0 (DCIS)	1.00	
	259	I	1.03 (0.50-2.13)	0.943
	115	IIA	1.65 (0.77-3.52)	0.197
	41	IIB	3.84 (1.70-8.65)	0.001
	5	IIIA	1.51 (0.19-11.87)	0.695

Multivariate Cox proportional hazards models of survival from date of diagnosis of breast cancer for breast cancer-free survival intervals were used to derive the adjusted relative hazards (RH) associated with each variable. Adjusted RH values were developed using p53 status (none, focal, low, high), TNM Stage (0 (DCIS), I, IIA, IIB, IIA), estrogen receptor (ER) status (present/absent), and TC group (≤ 200% / > 200%) as independent predictors of survival. The 95% confidence intervals for each RH are shown in parenthesis. Abbreviations: TC: Telomere DNA content, RH: Relative hazard, CI: Confidence Interval. See Materials and Methods Section for additional details.

Figure 1

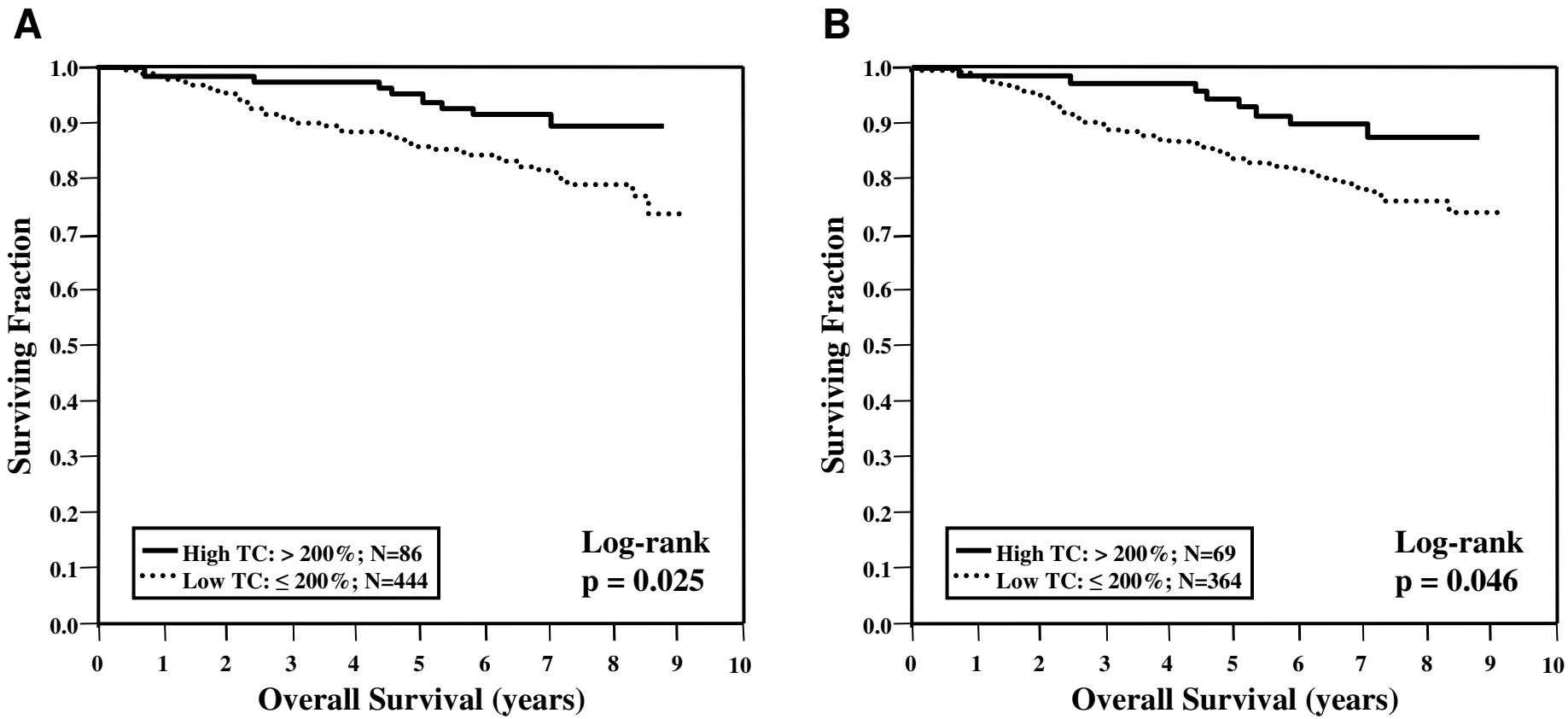
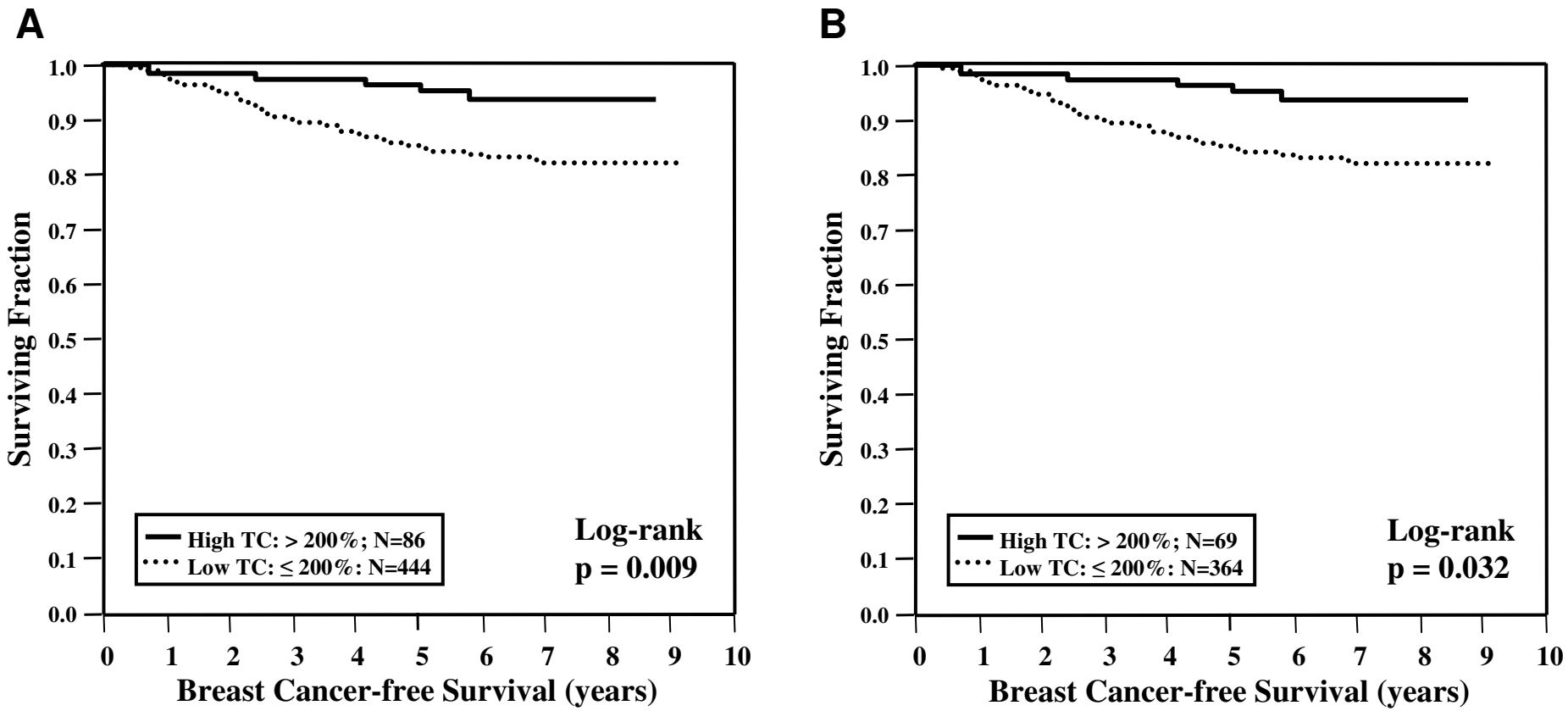

A
Surviving Fraction
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0 1 2 3 4 5 6 7 8 9 10
Overall Survival (years)
High TC: > 200%; N=86
Low TC: ≤ 200%; N=444
Log-rank
p = 0.025
B
Surviving Fraction
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0 1 2 3 4 5 6 7 8 9 10
Overall Survival (years)
High TC: > 200%; N=69
Low TC: ≤ 200%; N=364
Log-rank
p = 0.046

Figure 2


A
Surviving Fraction
1.0
0.9
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0.7
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0 1 2 3 4 5 6 7 8 9 10
Breast Cancer-free Survival (years)
High TC: > 200%; N=86
Low TC: ≤ 200%: N=444
Log-rank
p = 0.009
B
Surviving Fraction
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0 1 2 3 4 5 6 7 8 9 10
Breast Cancer-free Survival (years)
High TC: > 200%; N=69
Low TC: ≤ 200%: N=364
Log-rank
p = 0.032

Assessment of the Frequency of Allelic Imbalance in Human Tissue Using a Multiplex PCR System

Christopher M. Heaphy[1], William C. Hines[1,5], Kimberly S. Butler[1], Christina M. Haaland[1], Glenroy Heywood[2,4], Edgar G. Fischer[3], Marco Bisoffi[1,4] and Jeffrey K. Griffith[1,4]

Departments of [1]Biochemistry and Molecular Biology, [2]Surgery, [3]Pathology, and the [4]Cancer Research and Treatment Center, University of New Mexico School of Medicine, Albuquerque, New Mexico.

[5]Current Address: Division of Life Sciences, Lawrence Berkeley National Laboratory, Berkeley, California.

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Request for reprints: Jeffrey K. Griffith, Ph.D., Department of Biochemistry and Molecular Biology, MSC08 4670, 1 University of New Mexico, Albuquerque, New Mexico 87131-0001. Phone: 505-272-3444; Fax: 505-272-6587; Email: jkgriffith@salud.unm.edu

Abstract

Genomic instability can generate chromosome breakage and fusion randomly throughout the genome, frequently resulting in allelic imbalance, a deviation from the normal 1:1 ratio of maternal and paternal alleles. Allelic imbalance reflects the karyotypic complexity of the cancer genome. Thus, it is reasonable to speculate that tissues with more sites of allelic imbalance have a greater likelihood of having disruption of any of the numerous critical genes that cause a cancerous phenotype, and thus may have diagnostic or prognostic significance. For this reason, it is desirable to develop a robust method to assess the frequency of allelic imbalance in any tissue. To address this need, we designed an economical and high-throughput method, based on the Applied Biosystems AmpFℓSTR® Identifiler multiplex PCR system, to evaluate allelic imbalance at 16 unlinked, microsatellite loci located throughout the genome. This method provides a quantitative comparison of the extent of allelic imbalance between samples that can be applied to a variety of frozen and archival tissues. The method does not require matched normal tissue, requires little DNA (the equivalent of approximately 150 cells) and uses commercially available reagents, instrumentation and analysis software. Greater than 99% of tissue specimens with ≥ 2 unbalanced loci were cancerous.

Introduction

It is widely accepted that genomic instability- the duplication, loss or structural rearrangement of a critical gene(s) - occurs in virtually all cancers[1], and in some instances has diagnostic, prognostic or predictive significance. Thus, it is not surprising that tumor progression is reflected by allelic losses or gains in genes that regulate aspects of cell proliferation, apoptosis, angiogenesis, invasion and, ultimately, metastasis.[2,3]

There are several technologies available to detect chromosomal copy number changes in tumor cells. For example, chromosome painting techniques can identify chromosomal copy number changes in cytological preparations.[4,5] Segmental genomic alterations can be identified by comparative genomic hybridization (CGH). CGH identifies copy number changes by detecting DNA sequence copy variations throughout the entire genome and mapping them onto a cytogenetic map supplied by metaphase chromosomes.[6] Alternatively, array CGH maps copy number aberrations relative to the genome sequence by using arrays of BAC or cDNA clones as the hybridization target instead of the metaphase chromosomes.[7-11] However, these methods cannot identify all cases of allelic imbalance (AI), which is a deviation from the normal 1:1 ratio of maternal and paternal alleles, for instance in cases with uniparental disomy. Additionally, these methods are poorly suited for high-throughput applications and analysis is limited to a relatively small cellular field, thus increasing potential sampling error.

Single nucleotide polymorphism (SNP) arrays can be used for high-resolution genome-wide genotyping and loss of heterozygousity (LOH) detection.[12-14] For example, the

development of a panel of 52 microsatellite markers that detects genomic patterns of LOH [15-17] has been utilized for breast cancer diagnosis and prognosis. However, this approach requires matched referent (normal) DNA, typically blood or buccal samples, and these cancer-type specific panels may not be informative for other cancers, thus limiting their applicability across multiple tumor types. Larger panels of SNPs may be used for genome-wide analysis, for example the Affymetrix 10K and 100K SNP mapping arrays.[18-19] Likewise, Illumina BeadArrays with a SNP linkage-mapping panel,[20] allow allelic discrimination directly on short genomic segments surrounding the SNPs of interest, thus overcoming the need for high-quality DNA.[14] Lips and colleagues have shown that Illumina BeadArrays can be used to obtain reliable genotyping and genome-wide LOH profiles from formalin-fixed, paraffin-embedded (FFPE) normal and tumor tissues.[21] However, all these approaches, while robust, require costly reagents, specialized equipment, and the sheer amount of data produced from these analyses complicate the interpretation of results.

For these reasons, and as outlined by Davies et al.,[22] it is desirable to develop a general, economical, and high-throughput method to assess the frequency of AI in any tissue, independent of the nature and composition of the specimen and the availability of matched, normal tissue. To address this need, we developed a method to measure the ratio of maternal and paternal alleles at 16 unlinked, microsatellite short tandem repeat (STR) loci in a single multiplexed PCR reaction. The assay, which is based on the Applied Biosystems AmpFℓSTR® Identifiler system, can be performed with only 1 ng of genomic DNA, uses commercially available primers and reagents, and common

instrumentation and analysis software. Thus, it is an attractive alternative to current methods and is readily adaptable to most clinical laboratory environments.

Materials and Methods

Tissue Acquisition: All tissues were provided by the University of New Mexico Solid Tumor Facility, unless otherwise specified. Buccal cells were collected from oral rinses of volunteers. The Cooperative Human Tissue Network (Western Division, Nashville, TN) provided frozen normal and tumor renal tissues, obtained by radical nephrectomy, frozen normal breast tissues, obtained by reduction mammoplasty, and normal frozen prostate tissues, obtained through autopsy. A set of FFPE prostate tumors, obtained by radical prostatectomy, were provided by the Cooperative Prostate Cancer Tissue Resource (http://www.cpctr.cancer.gov). Duodenal FFPE tumor tissues were obtained from the Mayo Clinic (Rochester, MN). Pancreatic FFPE normal and tumor tissues were obtained from the Department of Pathology at the University of New Mexico. Frozen endometrial tumor tissues were obtained through the Gynecologic Oncology Group (Philadelphia, PA). All specimens lacked patient identifiers and were obtained in accordance with all federal guidelines, as approved by the UNM Human Research Review Committee.

DNA Isolation and Quantification: DNA was isolated from all tissue samples using the DNeasy® silica-based spin column extraction kit (Qiagen; Valencia, CA) and the manufacturer's suggested animal tissue protocol. FFPE samples were treated with xylene and washed with ethanol prior to DNA extraction. DNA concentrations were measured

using the Picogreen® dsDNA quantitation assay (Molecular Probes, Eugene, OR) using a λ phage DNA as the standard as directed by the manufacturer's protocol.

Multiplex PCR Amplification of STR Loci: The AmpFℓSTR® Identifiler kit (Applied Biosystems, Foster City, CA) was used to amplify genomic DNA at 16 different short tandem repeat (STR) microsatellite loci (Amelogenin, CSF1PO, D2S1338, D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51, D19S433, D21S11, FGA, TH01, TPOX and vWA) in a single multiplexed PCR reaction, according to the supplier's protocol. Linear amplification of allelic PCR products is a prerequisite for ratiometric determination of AI. Therefore, each PCR reaction was limited to 28 cycles, as determined in preliminary studies. The 16 primer sets are designed and labeled with either 6-FAM, PET, VIC or NED to permit the discrimination of all amplicons in a single electrophoretic separation. The PCR products were resolved by capillary electrophoresis using an ABI Prism® 377 DNA Sequencer (Applied Biosystems, Foster City, CA). Fluorescent peak heights were quantified using ABI Prism GeneScan® Analysis software (Applied Biosystems, Foster City, CA). Allelic ratios were calculated using the peak height, rather than the peak area, as suggested in previous studies.[23-25] For simplicity, the allele with the greater fluorescence was always made the numerator, as to always generate a ratio ≥ 1.0.

Statistical Analysis: A Pearson Chi-square test was performed using SAS JMP® software version 9.1 (SAS Institute Inc., Cary, NC) to examine the relationship between the extent of AI and tissue type, using a significance level of 0.05.

Results

The 16 allelic microsatellite loci amplified by the AmpFℓSTR® Identifiler primer sets are unlinked, and can be used to assess AI simultaneously at multiple heterozygous sites throughout the genome. This is technically possible because each amplicon is labeled with one of four fluorescent dyes (6-FAM, PET, VIC and NED), each with a unique emission profile, thus allowing the resolution of amplicons of similar size. Figure 1 shows the sizes of VIC-labeled amplicons derived from a representative specimen of matched normal and tumor renal tissue (the fluorescent channels showing the PET, 6-FAM, and NED-labeled products are not shown). Within Figure 1A, illustrating the results from the normal tissue specimen, two of the allelic pairs are homozygous (D13S317, D16S539), as indicated by a single peak, and three of the allelic pairs are heterozygous (D3S1358, TH01, D2S1338), as indicated by two peaks. Although the peak heights varied between different loci, ostensibly due to different PCR efficiencies, the peak heights of the paired alleles were similar. Theoretically, the ratio of any two heterozygous alleles is 1.0 in normal tissues. However, differences in PCR efficiency between different length alleles and random experimental variation resulting from instruments, reagents and personnel may affect the observed ratio of heterozygous alleles. To assess these sources of potential variation, the ratios of paired alleles' signal intensities were compared at 320 heterozygous loci in buccal cells from 27 healthy individuals. Across all loci, the mean ratio was near 1.0 (mean =1.15, SD 0.18). We expect that approximately 97.5% of all allelic ratios in normal tissues would fall within 2.5 SD of the mean, and therefore operationally defined an allelic ratio of >1.60 (mean + 2.5 SD) as a site of AI. Applying this threshold to the 27 analyzed buccal samples, only 8

sites of AI were detected out of the 320 heterozygous loci, thus representing a mean of 0.30 unbalanced loci per sample. Figure 1B illustrates the results of the tumor tissue matched to the normal sample in Figure 1A. Within this sample, two of the three heterozygous loci in the renal tumor tissue amplified by the VIC-labeled primer sets have peak height ratios of >1.60, identifying them as sites of AI.

To determine whether AI determinations were reproducible, the assay was repeated within a random subset of the buccal samples. The mean absolute variation of the allelic ratios for the repeated samples was 10% and 193 of the 198 (97.5%) loci measured were correctly categorized upon repeating the experiment; whereas, only 5 of the 198 (2.5%) loci initially designated as sites of AI could not be confirmed (Figure 2A). Two loci changed from sites without AI (≤ 1.60) to sites of AI (> 1.60) and three loci changed from sites of AI to sites without AI.

We next confirmed that the differences in AI detected by this approach reflected true differences in the ratio of the alleles, and not experimental artifact (e.g. differential PCR amplification efficiency), we constructed defined mixtures of DNAs from the paired normal and tumor tissue shown in Figure 1. As shown in Figure 2B for the D3S1358 locus, there was a linear relationship (R^2=0.965) between the ratio of alleles measured in the assay and the composition of the mixture. Similar results were obtained for each of the other loci exhibiting a site of AI (TH01: R^2=0.973; VWA: R^2=0.981; D18S541: R^2=0.953). In contrast, the composition of the mixture had no effect on the allelic ratios of loci not exhibiting AI (data not shown).

The operationally-defined threshold for AI was validated by measuring the allelic ratios for 1382 heterozygous loci in an independent test set comprised of 118 normal samples consisting of bone (n=2), breast (n=10), buccal (n=53), lymph node (n=5), peripheral blood lymphocytes (PBL) (n=18), pancreas (n=6), placenta (n=3), prostate (n=4), renal (n=16) and tonsil (n=1) tissues (Figure 3A). In this sample set of normal tissues, only 32 of 1382 heterozygous loci were designated sites of AI, thus representing a mean of 0.27 unbalanced loci per sample, comparable to the 0.30 unbalanced loci per sample in the original normal sample set. In summary, 88 (74.6%), 29 (24.6%), and 1 (0.8%) of the 118 normal tissues specimens contained 0, 1 and 2 loci with AI, respectively.

It is well established that cancerous tissues have more sites of AI than normal tissues. To validate our assay in this context, we next measured the frequency of AI in 2792 heterozygous loci in a set of 239 frozen or FFPE tumor samples consisting of AML (n=8), breast (n=39), CML (n=3), duodenal (n=23), endometrial (n=78), pancreas (n=6), prostate (n=47), and renal (n=35) tissues. As shown in Figure 3B, 37 (15.5%), 41 (17.2%), and 161 (67.4%) of the 239 tumor tissues specimens contained 0, 1 and ≥ 2 loci with AI, respectively. In contrast to the normal tissues, 611 sites of AI were detected, thus representing a mean of 2.56 unbalanced loci per sample, nearly 10 times greater than the frequency in the normal tissues ($p < 0.0001$). In summary, 162 of 357 tissue specimens had ≥ 2 unbalanced loci, of which >99% were cancerous.

Discussion

The frequency of AI reflects the karyotypic complexity of the cancer genome and such manifestations are widespread in solid tumors.[1] There have been numerous studies of these abnormalities and several techniques, including chromosome painting, array CGH and SNP arrays, have emerged to analyze these differences between normal and tumor tissues.[4-21] However, these methods are typically costly, time intensive, and need a matched referent (normal) DNA sample for analysis. For this reason, it is desirable to develop general, economical, high-throughput methods to quantify the extent of AI in the genome of any tissue, independent of the nature and composition of the specimen and the availability of matched, normal tissue.

Using our newly developed assay and interpretation scheme to assess the frequency of AI in human tissues, we have shown in a set of 239 samples that 67% of the tumors contained two or more sites of AI, as compared to 0.8% of the normal samples, which represents an almost 84 fold difference. It must be noted that tissue heterogeneity, such as a proponderance of normal cells within the tumor, may quench peak-height ratios below the 1.60 threshold, thus obscuring allelic imbalance in a particular sample. Additionally, the assay cannot discriminate between homozygous alleles and complete loss of heterozygosity in the absence of matched normal tissue. However, the latter limitation is mitigated by the near ubiquitous presence of normal tissue within tumors which allows for the assessment of AI in samples without requiring analysis of matched normal tissue. This is an important consideration in the potential evaluation of biopsy tissue, which may

contain multiple clones of genetically altered cells superimposed on a background of normal stromal and epithelial cells and obtaining matched normal tissue may be difficult.

Altered gene expression resulting from genomic instability is a cause of cancer progression; therefore, cancerous tissues have more sites of AI than normal tissues. Consistent with this observation, >99% of tissues with ≥2 sites of AI were cancerous. We are currently investigating the possibility that the *number* of sites of AI in cancer tissue is a reflection of its stage of progression, and therefore may be correlated with clinical parameters or prognosis.

Existing alternative methods identify AI as a difference in the allelic ratios in the sample of interest (e.g. tumor) *relative to* the allelic ratios in a patient-matched referent DNA. These methods allow for the distinction between complete LOH and a constitutive homozygous allele and are able to control for PCR efficiency differences of alleles of dissimilar length. In contrast, the present method identifies AI as a deviation from a 1:1 ratio between alleles *within the sample of interest only*. Thus, the assay described herein can be performed on specimens for which a reliable referent sample is not available. Additionally, we have determined that the mean absolute variation of the allelic ratios for all microsatellite loci in our panel is approximately 15% in normal tissues. This variation represents the combined effects of (i) random experimental error resulting from instruments, reagents and personnel, (ii) copy-number polymorphisms, and (iii) inherent differences in the PCR efficiencies of microsatellite alleles of dissimilar lengths. Based on replicate experiments of the same sample (Figure 2A), we have determined that

random experimental variation resulting from instruments, reagents and personnel accounts for approximately 10% of the overall variation. Therefore, variation resulting from differences in PCR efficiencies is approximately 5%. Although the latter variation is excluded by comparison to a referent DNA, the requirement for two determinations (sample of interest and referent), each with an average variation of at least 10%, minimizes the benefit gained by controlling for PCR efficiency.

In conclusion, we describe here a simple method for assessing the extent of AI throughout the genome. This method has a number of significant advantages over existing technologies, such as chromosome painting, array CGH and SNP arrays, and as a molecular based assay may be utilized clinically in conjunction with histological techniques. The advantages of this method are that: (i) it is robust, reproducible and provides a quantitative basis for comparing the extent of AI between samples; (ii) it does not require matched normal tissue; (iii) it utilizes commercially available reagents, instrumentation and analysis software; (iv) it can be applied to a variety of fresh, frozen and archival tissues; (v) it requires very little DNA (the equivalent of approximately 150 cells); and (vi) >99% of tissues with ≥2 sites of AI were cancerous.

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Figure Legends

Figure 1. Electropherograms of VIC-labeled amplicons from a matched normal and renal carcinoma sample. PCR was performed and the resulting amplicons resolved as described in Materials and Methods. Only VIC-labeled amplicons are shown. In this particular sample, the D3S1358, THO1 and D2S1338 loci are heterozygous and D13S317 and D16S539 loci are homozygous. Fluorescent intensity is shown on the y-axis and amplicon size, in base pairs, is shown on the x-axis. The ratios of the fluorescent intensities of each allelic pair of heterozygous loci are shown. Loci with allelic ratios of >1.60 are defined as sites of allelic imbalance for matched normal (A) or tumor (B) tissue.

Figure 2. Reproducibility and effect of admixtures of matched normal and renal carcinoma DNA on allelic peak height ratios. (A) Allelic peak height ratios were determined for 198 heterozygous loci in16 normal buccal samples. The plot represents the first determination (x-axis) and the second determination (y-axis). The region defined by the gray shaded box represents all the loci that were determined not to be a site of AI on both determinations. The labeled points (allelic peak height ratios for both determinations) represent the five loci that were not correctly identified upon repeating the experiment. (B) The specified admixtures were generated using DNA from a matched pair of normal renal tissue and renal cell carcinoma as shown in Figure 1. Data from the heterozygous D3S1358 locus are shown. The allelic ratios are 1.09 in the normal renal tissue and 2.02 in the renal carcinoma. The best-fit line was generated by linear regression and has a correlation coefficient (R^2) of 0.965.

Figure 3. Frequency of allelic imbalance in normal and tumor tissues. The numbers of sites of allelic imbalance (i.e. 0, 1, ≥2) were determined in 118 samples of normal tissue (A) and in 239 samples of tumor tissue (B). The number of specimens in each tissue set (n) is indicated below the set designation. Abbreviations: Lymph Node: LN; Peripheral Blood Lymphocytes: PBL; Acute Myelogenous Leukemia: AML; Chronic Myelogenous Leukemia: CML; Endometrial: Endo. See Materials and Methods for additional details.

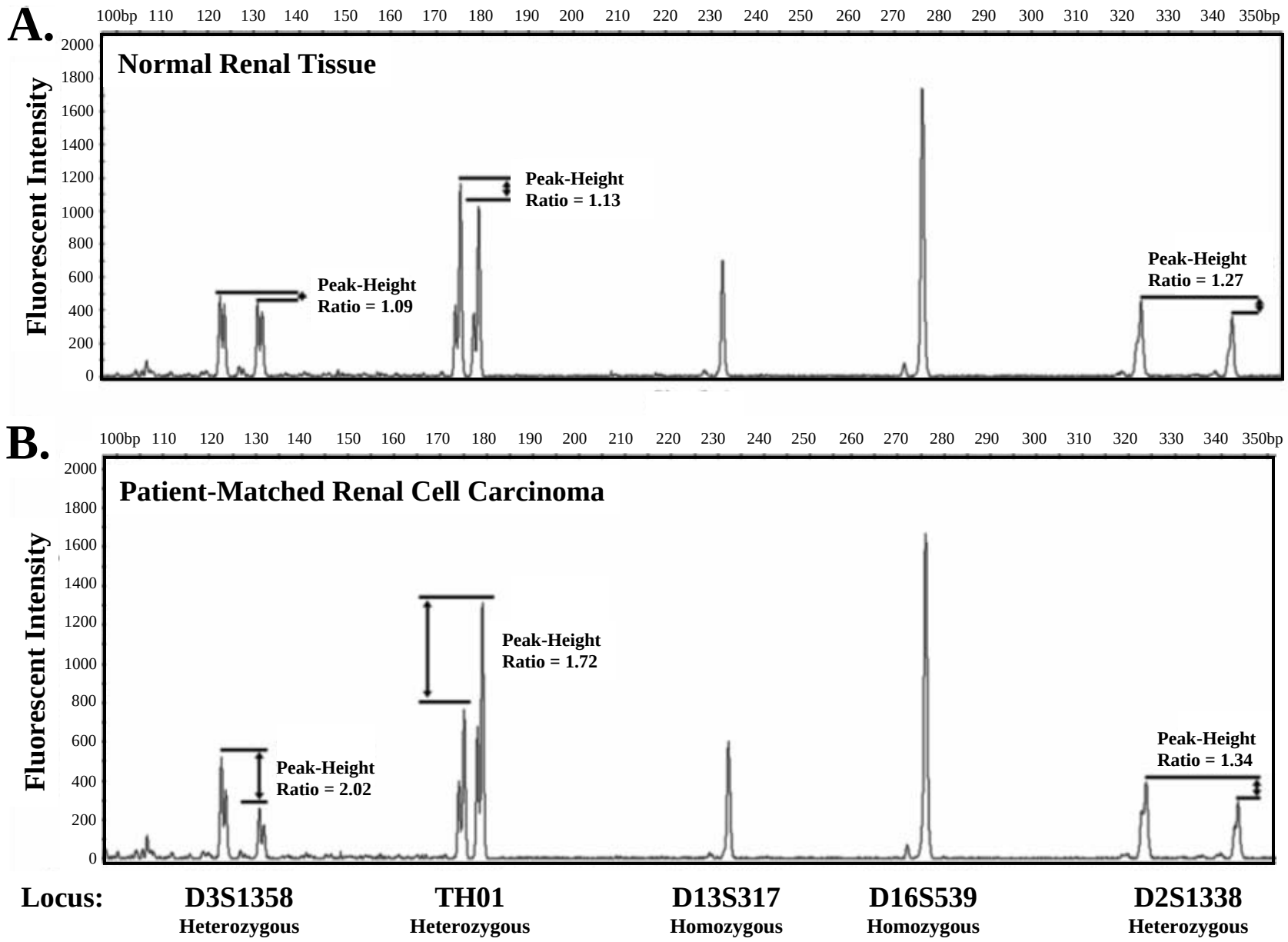
A.
100bp 110 120 130 140 150 160 170 180 190 200 210 220 230 240 250 260 270 280 290 300 310 320 330 340 350bp
Fluorescent Intensity
2000 1800 1600 1400 1200 1000 800 600 400 200 0
Normal Renal Tissue
Peak-Height Ratio = 1.09
Peak-Height Ratio = 1.13
Peak-Height Ratio = 1.27
B.
100bp 110 120 130 140 150 160 170 180 190 200 210 220 230 240 250 260 270 280 290 300 310 320 330 340 350bp
Fluorescent Intensity
2000 1800 1600 1400 1200 1000 800 600 400 200 0
Patient-Matched Renal Cell Carcinoma
Peak-Height Ratio = 2.02
Peak-Height Ratio = 1.72
Peak-Height Ratio = 1.34
Locus: D3S1358 TH01 D13S317 D16S539 D2S1338
Heterozygous Heterozygous Homozygous Homozygous Heterozygous

Figure 1

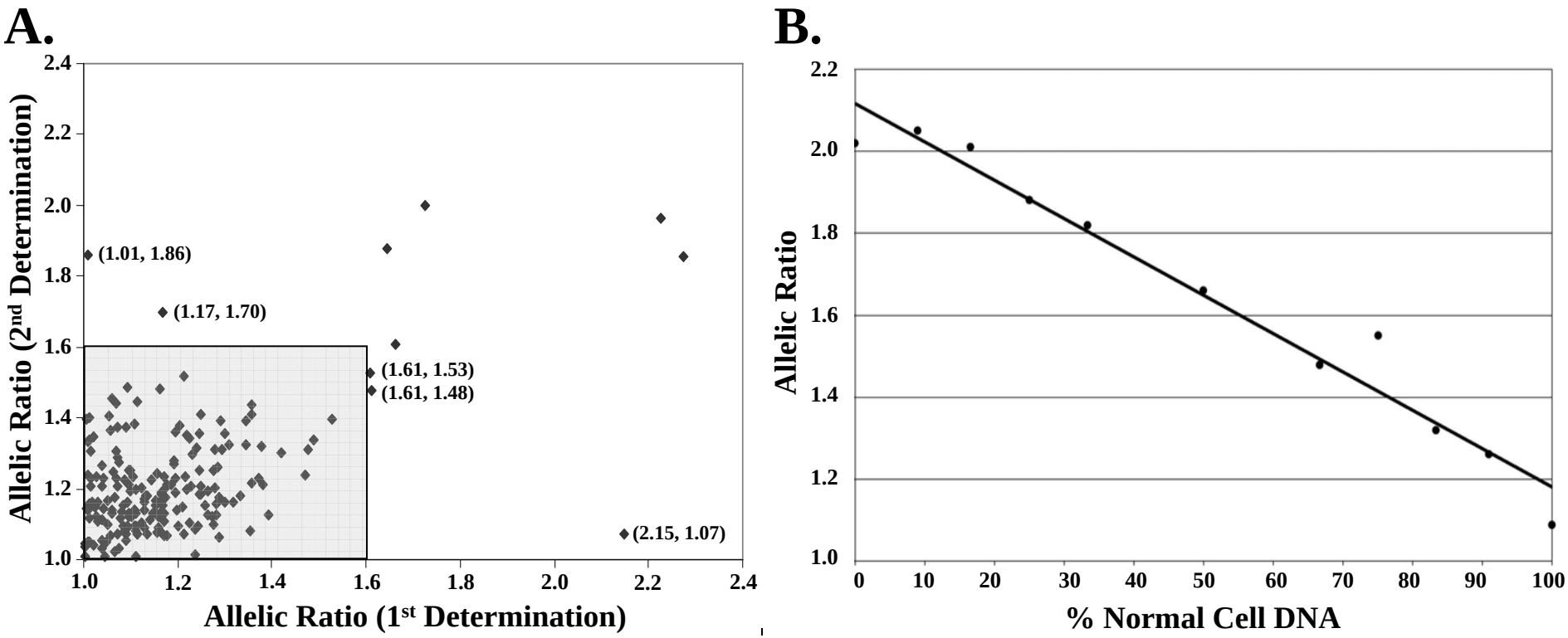
A.
B.
Allelic Ratio (2nd Determination)
Allelic Ratio (1st Determination)
(1.01, 1.86)
(1.17, 1.70)
(1.61, 1.53)
(1.61, 1.48)
(2.15, 1.07)
Allelic Ratio
% Normal Cell DNA

Figure 2

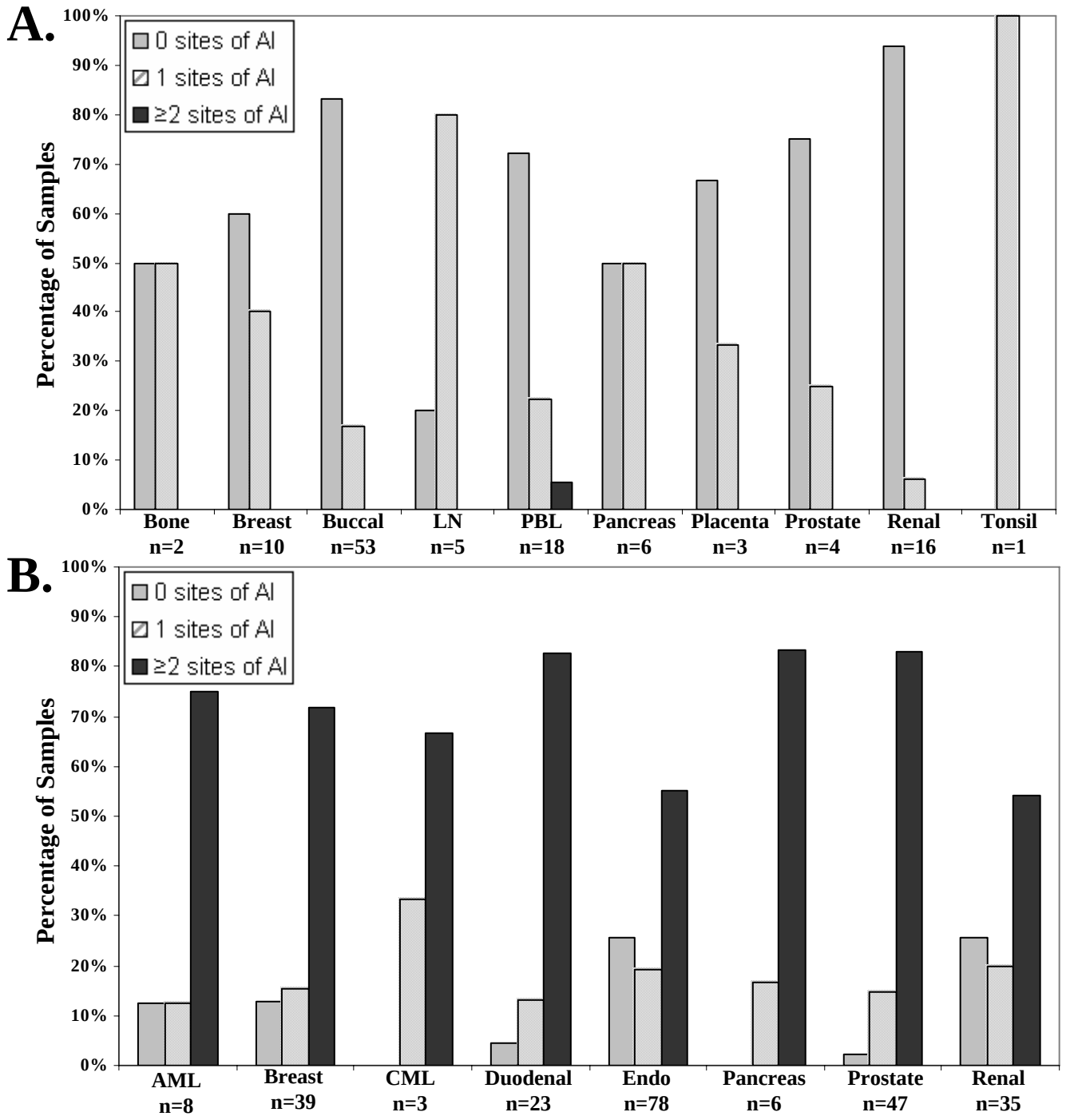
A.
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Percentage of Samples
0 sites of AI
1 sites of AI
≥2 sites of AI
Bone n=2
Breast n=10
Buccal n=53
LN n=5
PBL n=18
Pancreas n=6
Placenta n=3
Prostate n=4
Renal n=16
Tonsil n=1
B.
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Percentage of Samples
0 sites of AI
1 sites of AI
≥2 sites of AI
AML n=8
Breast n=39
CML n=3
Duodenal n=23
Endo n=78
Pancreas n=6
Prostate n=47
Renal n=35

Figure 3

Telomere DNA Content in Prostate Biopsies Predicts Early Rise in Prostate Specific Antigen Following Radical Prostatectomy for Prostate Cancer

Eric G. Treat[1,2,‡], Christopher M. Heaphy[3,‡], Larry W. Massie[6], Marco Bisoffi[3,4], Anthony Y. Smith[1,4], Michael S. Davis[1,5], Jeffrey K. Griffith[1,3,4]

[1]Department of Surgery, Division of Urology, University of New Mexico School of Medicine, Albuquerque, New Mexico
[2]General Clinical Research Center, University of New Mexico Hospital, Albuquerque, New Mexico
[3]Department of Biochemistry and Molecular Biology, University of New Mexico School of Medicine, Albuquerque, New Mexico
[4]Cancer Research and Treatment Center, University of New Mexico School of Medicine, Albuquerque, New Mexico
[5]Department of Surgery, Division of Urology, Veteran's Affairs Medical Center in Albuquerque, New Mexico
[6]Department of Pathology, Veteran's Affairs Medical Center in Albuquerque, New Mexico

Running Title: Prognostic Value of Telomere DNA Content in Prostate Biopsies

Key Words: prostate adenocarcinoma, genomic instability, prognosis, telomere, PSA recurrence

Corresponding Author: Jeffrey K. Griffith, Ph.D., Department of Biochemistry and Molecular Biology, MSC 08 4670, 1 University of New Mexico, Albuquerque, New Mexico 87131-0001. Phone: (505) 272-3444; Fax: (505) 272-6587; E-mail: jkgriffith@salud.unm.edu

[‡]Eric G. Treat and Christopher M. Heaphy contributed equally to this study.

ABSTRACT

Purpose: To determine whether measurement of telomere DNA content in prostate biopsy tissue predicts PSA recurrence in men after undergoing radical prostatectomy for prostate cancer.

Materials and Methods: Slot blot titration assay was used to quantitate telomere DNA content in archived diagnostic prostate needle biopsy specimens for subjects (n=103) diagnosed with prostate cancer and subsequently undergoing radical prostatectomy between 1993 and 1997. Telomere DNA content was compared to the clinical outcome measure, PSA recurrence, defined as an increase in PSA ≥0.4 ng/mL on two or more consecutive measurements post-prostatectomy, observed retrospectively, for a mean follow-up period of 114 months (range 1-165).

Results: In the cohort, 47 subjects had a PSA recurrence. In a univariate Cox proportional hazards model, low telomere DNA content (<0.3 of standard) demonstrated a significant associated risk for PSA recurrence (HR=2.31; 95% CI:1.22-4.34, p=0.0097). In a multivariate Cox proportional hazards model, low telomere DNA content was also significantly associated with PSA recurrence after controlling for pre-operative PSA levels and biopsy Gleason sum (HR=2.56; 95% CI:1.23-5.32, p=0.012). In a subset analysis of men with pathologic Gleason sum ≤7 (n=84; 28 recurrences), low telomere DNA content demonstrated greater risk of PSA recurrence (HR=4.11; 95% CI:1.92-8.81, p=0.00027), even after adjusting for pre-operative PSA level and biopsy Gleason sum (HR=8.31; 95% CI:3.26-21.5, p<0.0001).

Conclusion: Low telomere DNA content measured in prostate biopsy tissue predicts early likelihood of post-prostatectomy PSA recurrence independent of pre-operative PSA level and biopsy Gleason sum in a retrospective analysis, particularly in men with Gleason sum ≤7 disease.

INTRODUCTION

In the modern era of prostate-specific antigen (PSA) testing, there has been a steady annual decrease in the number of men presenting with advanced disease compared with an increased number of localized prostate cancers detected[1]. This shift to increased localized disease detection has caused some confusion and debate over appropriate screening and management strategies, as well as generating a demand for clinicians and basic scientists to develop more refined techniques for determining the prognosis of prostate adenocarcinoma. A number of pretreatment prognostic tools may help predict disease outcome for patients with localized prostate cancer. These include: pre-operative PSA, tumor grade by Gleason sum at biopsy, and clinical stage by the Tumor-Node-Metastasis (TNM) model developed by the American Joint Committee on Cancer (AJCC). Pathological stage, the most reliable predictor of tumor progression, recurrence and overall disease outcome, is only available if a patient chooses to undergo radical prostatectomy. Several limiting factors surrounding the use of these TNM staging and common clinical tumor markers argue for the development of more reliable prognostic markers prior to therapeutic intervention. To meet this need, efforts have focused on development of molecular and genetic markers available at the time of biopsy.

It is well established that genomic instability, *i.e.* the amplification, loss or structural rearrangement of a critical gene or genes, occurs in virtually all cancers, including prostate cancer[2]. Telomere dysfunction is one mechanism that generates genomic instability[3, 4]. Recently, our laboratory demonstrated that a decrease in relative telomere DNA content, a proxy for telomere length[5, 6], in prostate cancer cells and adjacent histologically normal appearing tissue sampled from radical prostatectomy specimens, independently predicts prostate cancer recurrence[7, 8]. Therefore, measurement of telomere DNA content in prostate biopsy tissue could potentially give important prognostic information prior to undergoing invasive treatments (i.e. radical prostatectomy). The purpose of the present study is to determine retrospectively whether telomere DNA content measured in prostate biopsy tissue predicts PSA recurrence in men treated by radical prostatectomy.

MATERIALS AND METHODS

Study population. This study was conducted according to a protocol approved by the University of New Mexico Human Research Review Committee and the Veteran's Administration Research and Development Committee. All men who underwent standard clinical evaluation and treatment for prostate cancer with a perineal or retropubic radical prostatectomy without first receiving neoadjuvant radiation or hormone therapy from 1993 through 1997 at the Veteran's Affairs Medical Center (VAMC) in Albuquerque, NM, were identified retrospectively in the electronic medical record database to ensure an adequate mean follow-up period of 10

years. Of the 125 subjects identified, men were excluded: (i) who did not have a diagnostic biopsy performed at the VAMC (*n*=10), (ii) were diagnosed by transurethral resection of the prostate (*n*=5), (iii) were determined to have pathological Tx disease (*n*=3), or (iv) were missing archived biopsy core tissue (*n*=4), leaving a total of 103 subjects. All subjects had prostate adenocarcinoma pathology at the time of pathological evaluation of the prostate biopsy.

Clinical data. Clinical data were individually reviewed and abstracted from the electronic medical record and entered into a separate, de-identified database. Abstracted data included: age at diagnosis, race/ethnicity, pre-treatment PSA measurements (ng/mL), biopsy Gleason sum according to the original clinical pathology biopsy report, pathological stage (expressed as a binary variable defined as "organ confined" if pT2c or less) and grade ("pathological Gleason sum"), surgical margin status, post-treatment PSA measurements, neoadjuvant and adjuvant prostate cancer specific treatments, and cause of death, where applicable. The date of the biopsy-confirmed diagnosis was used as the follow-up period starting point and the most recent dated clinical record or date of death was used to define the follow-up end point. We defined PSA recurrence to be a post-radical prostatectomy PSA ≥0.4 ng/mL on at least 2 consecutive tests more than one year after their radical prostatectomy. Men were considered to have progression (also termed "PSA recurrence" in this study) if they had a post-radical prostatectomy PSA ≥0.4 ng/mL on at least 2 consecutive tests less than one year after their radical prostatectomy or if they underwent adjuvant treatment within one year of radical prostatectomy despite no PSA elevation. The laboratory determining the telomere DNA content of the biopsy tissues and the clinical data abstractors were blinded until all the data were independently collected and confirmed.

Determination of telomere DNA content. 100 μm of prostate biopsy core tissue was sampled in serial 25 μm sections from archived paraffin blocks for each subject. Prostate cancer was not microdissected from the core. Embedded tissue was extracted from the paraffin using xylene and washed in ethanol prior to DNA isolation with the DNeasy® Tissue Kit (Qiagen, Valencia, CA) and telomere DNA content was determined for all the study subjects in triplicate by the chemiluminescent slot blot assay as previously described[5, 6]. Telomere DNA content for each sample is reported as the ratio of the telomere DNA content in the representative sample to a placental DNA control and therefore reported without units. The intra-sample mean coefficient of variation was 27%.

Statistical methods. Data were analyzed in a fashion consistent with the recently published recommendations for tumor marker prognostic studies[9]. Welch's student t-tests for continuous variables and χ^2 or Fisher's exact tests for categorical variables were used for univariate comparisons between non-recurrent and recurrent groups.

Survival distributions were estimated using the Kaplan-Meier method, and compared with log-rank tests. Subjects were censored if lost to follow-up or deceased prior to any detectable rise in PSA levels. Hazard ratios (HR) and 95% confidence intervals (95% CI) were determined using Cox proportional hazards models. Multivariate approaches were developed in a manual hierarchical approach based on the likelihood ratio test and changes in the magnitude of hazard ratios. Simple linear regression models and correlation coefficients were used for all correlations. Statistical analyses were performed using R statistical software version 1.14 from the R Foundation for Statistical Computing©.

RESULTS

Telomere DNA content and prediction of PSA recurrence. Of the 103 men who underwent radical prostatectomy, 71 (68.9%) men remained alive, 27 (26.2%) men died, and 5 (4.9%) men were lost to follow-up over a mean follow-up period of 114 months (range 1-165). **Table 1** lists the population characteristics of the study population comparing those without detectable PSA rise post-prostatectomy versus those with a PSA recurrence for all the study subjects, as well as a subset analysis of men who had a pathological Gleason sum of 7 or less. During the follow-up period, 45 (43.7%) of all study subjects had a PSA recurrence. Overall, there was no significant difference in the mean age at diagnosis, biopsy Gleason sum or mean biopsy telomere DNA content for all study subjects when comparing men who experienced PSA recurrence versus those who did not. The mean highest measured pre-operative PSA value in the recurrence group, however, significantly differed from the non-recurrence group (mean of 7.96 ng/mL ±SE of 1.16 and mean of 11.33 ng/mL ±SE of 0.80, respectively, p=0.031). In the subset analysis, the mean highest pre-operative PSA no longer significantly differed between recurrence and non-recurrence groups, whereas, mean telomere DNA content became significantly different (mean of 1.21 ±SE of 0.09 and mean of 0.82 ±SE of 0.08, p=0.035, respectively). This finding indicates that telomere DNA content optimally predicts PSA recurrence in men with lower grade disease where elevated pre-operative PSA tends to correspond with higher grade disease in predicting rise in post-operative PSA. As expected, the post-operative prognostic variables of pathological Gleason sum, organ confined disease (pathological stage), seminal vesicle invasion, and surgical margin involvement, in the recurrence group were significantly higher than the non-recurrence group. In the subset of men, these differences remained significant except for pathological grade, which represents a subset effect.

Univariate analysis of telomere DNA content and PSA recurrence. **Table 2** lists the Cox proportional hazards models for telomere DNA content and other prognostic variables with respect to time (in months) to event of post-prostatectomy PSA recurrence for all study subjects and the subset of men with pathological Gleason sum 7 or less. The lowest quintile (<0.3) was used as the cutoff point for the telomere DNA content variable, while

other commonly utilized cutoffs were used for the other markers. When examining all study subjects, significant associations were seen with low (<0.3) telomere DNA content (HR 2.31; 95% CI, 1.22-4.34, p=0.0097), a pre-operative PSA value ≥10.0 ng/mL (HR 3.47; 95% CI, 1.18-10.17, p=0.023), and biopsy Gleason sum ≥8 (HR 2.63; 95% CI, 1.24-5.55, p=0.011) in predicting early PSA recurrence. Post-prostatectomy variables were all significantly associated with early PSA recurrence. Organ confined disease was associated with decreased risk of early PSA recurrence (HR 0.33; 95% CI 0.18-0.60, p=0.00032), whereas seminal vesicle invasion, or involvement of the surgical margin all were significantly associated with increased risk of early PSA recurrence. In the subset analysis, only telomere DNA content remained a significant predictor of early PSA recurrence. In this context, telomere DNA content actually improved in significant predictive ability (HR 4.11; 95% CI, 1.92-8.81, p=0.00027), while pre-operative PSA and biopsy Gleason sum both lost significant predictive power. Of note, in this subset of men, the post-operative variable of seminal vesicle involvement was no longer significant, but is likely a reflection of the small number of men with seminal vesicle involvement in the subset analysis. All of the other post-operative variables significantly differed between the two groups.

Kaplan-Meier survival analysis of telomere DNA content and PSA recurrence. For all subjects, Kaplan-Meier survival analysis for time to PSA recurrence following radical prostatectomy in regards to telomere DNA content demonstrated significantly different PSA recurrence-free survival curves (survival rate for men with telomere DNA content ≥0.3 of 59% ±SE of 6% and telomere DNA content <0.3 of 33% ±SE of 10%, respectively; log-rank p=0.0079; **Figure 1a**). Pre-operative PSA showed significant PSA recurrence-free survival when using high-risk cutoffs (10-year survival rate for men with PSA <10 ng/mL of 63% ±SE of 6% and for PSA ≥10.0 ng/mL 37% ±SE of 9%, log-rank p=0.017; **Figure 1b**). Additionally, Gleason sum showed significant PSA recurrence-free survival when using a high-risk cutoff (10-year survival rate for men with biopsy Gleason sum <8 of 57% ±SE of 6% and for Gleason sum ≥8 of 33% ±SE of 12%, log-rank p=0.013; figure not shown). Alternatively, in the subset analysis of men with pathological Gleason sum 7 or less disease, the PSA recurrence-free survival curves widened further for telomere DNA content (survival rate for men with telomere DNA content ≥0.3 of 71% ±SE of 6% and telomere DNA content <0.3 of 31% ±SE of 12%, respectively; log-rank p=0.000080; **Figure 1c**). Additionally, pre-operative PSA lost significant association with PSA recurrence-free survival when using high-risk cutoffs (10-year survival rate for men with PSA <10 ng/mL of 70% ±SE of 6% and for PSA ≥10.0 ng/mL 53% ±SE of 11%, log-rank p=0.017; **Figure 1d**). These models strongly support the hypothesis that low telomere DNA content predicts early PSA recurrence. Specifically, low telomere DNA content optimally predicts early rise in PSA in men with intermediate to low grade disease, helping to separate this clinically homogenous group into men with high and low risk of PSA recurrence post-prostatectomy prior to undergoing any treatment.

Multivariate analysis of telomere DNA content and PSA recurrence. A multivariate Cox proportional hazards model for telomere DNA content and the other prognostic variables with respect to time (in months) to event of post-prostatectomy PSA recurrence for all study subjects and the subset of men with pathological Gleason sum 7 or less is shown in **Table 3**. The association of low telomere DNA content to post-prostatectomy PSA recurrence when adjusted for pre-operative variables (highest pre-operative PSA value and biopsy Gleason sum) was significant (HR 2.56; 95% CI 1.23-5.32, p=0.012) for all study subjects. Pre-operative PSA above 10 ng/mL also conferred significant risk (HR 4.39; 95% CI 1.28-15.6, p=0.022). Both of these variables remained significant in the subset analysis; moreover, the overall hazard for low (<0.3) telomere DNA content (HR 8.31; 95% CI 3.26-21.5, p<0.0001) became larger than a PSA value above 10 ng/mL (HR 5.13; 95% CI 1.23-21.3, p=0.024). There were no significant interactions or associations of low (<0.3) telomere DNA content and the other prognostic markers in this study (**Table 4**). No correlations were seen in regression models of telomere DNA content compared to highest pre-operative PSA and biopsy Gleason sum (multiple R^2=0.0018, p=0.68 and multiple R^2=0.00073, p=0.79, respectively). When controlling for biopsy Gleason sum and pre-operative PSA, low telomere DNA content measured in biopsy samples significantly predicted early PSA recurrence, demonstrating a potential clinical role as an independent pre-operative prognostic marker.

DISCUSSION

In the present study, we show that telomere DNA content measured in the prostate biopsy specimen, independent of other pre-operative variables, predicts early PSA recurrence after radical prostatectomy. Men who had a telomere DNA content ratio less than 0.3 were at significant risk of having an earlier rise in their PSA, suggesting recurrence of their disease despite definitive surgical treatment. This study confirms our previous work in which telomere DNA content in prostatectomy tissue independently predicted PSA recurrence[7], and extends the hypothesis to suggest telomere DNA content measured in biopsy tissue, can be used as a predictive marker for radical prostatectomy outcome along with other established and clinically available prognostic markers, such as PSA and Gleason sum. In this study, telomere DNA content predicted recurrence in men who had intermediate to low grade tumors better than in men with high grade disease. This unique predictive quality of telomere DNA content could help many patients faced with the challenge of weighing the risks and benefits of surgical treatment when they are diagnosed with low to intermediate grade prostate cancer.

Telomeres are specialized protein-nucleic acid structures that protect the ends of eukaryotic chromosomes from degradation and recombination, and due to incomplete replication are shortened during each round of cellular

replication. Shortened, dysfunctional telomeres are prone to chromosome fusion and breakage. In normal somatic cells this leads to timely p53-dependent senescence and apoptosis[10, 11]. In cancer cells, these mechanisms are inactivated, and if unchecked, the accumulation of these chromosomal aberrations is lethal. Consequently, stabilization of telomeres, through activation of telomerase, is essential for tumor progression[12, 13]. The cause-and-effect relationship between dysfunctional telomeres and genomic instability implies that tumors with the shortest telomeres have the least stable genomes, and thus, have the greatest probability of containing cells capable of invasion, extravasation and metastasis. Likewise, tumors with the longest telomeres would be expected to have fewer genomic alterations, and therefore, lower probability of containing cells with the phenotypes associated with disease recurrence. Consistent with this view, numerous studies have associated reduced telomere length with poor clinical outcome or markers of disease progression[14].

Whether telomere DNA content can predict significant morbidity or mortality from prostate cancer is not known. This study was not specifically designed to address clinical progression and survival. PSA recurrence may not be a valid surrogate endpoint for radical prostatectomy outcome and disease-free survival[15]; however, the use of PSA recurrence makes an initial and compelling argument to further investigate telomere DNA content in this context.

Prior studies indicate that telomere shortening occurs in precursor lesions, preceding histological changes associated with complete malignant transformation[16-18]. Likewise, in our previous study, telomere DNA content in tumor tissue correlated to matched, histologically normal adjacent tissue suggesting a “field effect”, or “field cancerization” in the surrounding tissue[7]. The distance from the tumor tissue to which the field extends is not known or well characterized. Therefore, a biopsy core containing a vector sample of tissue with only a fraction of adenocarcinoma may be a mixture of normal tissue telomere lengths and shortened preneoplastic and/or neoplastic telomere lengths, inadvertently increasing or decreasing the observed telomere DNA content ratio. The fact that we were able to discern a difference in outcome without microdissection, despite a possible “field effect”, attests to the robustness of this particular telomere DNA assay.

This study attempted to pre-define and include all men undergoing radical prostatectomy in a certain time period to reduce the potential limitations and biases of a retrospective analysis. The exclusion and lost-to-follow-up rate is extremely low, strengthening the validity of these results. Only a well-designed prospective study would completely resolve these limitations. Moreover, the prostatic telomere DNA content distribution in men unaffected by prostate cancer is not well described. In order to make this assay clinically applicable, a validated, inter-laboratory assay with well-described reference ranges needs to be developed. In agreement with our previous studies[7, 8], this study continues to support the hypothesis that decreased telomere length induces

genomic instability and increased mutation rates which, in turn, lead to more aggressive tumors with elevated growth rates and/or metastatic capability. Nonetheless, the mechanisms by which low telomere DNA content confers risk of PSA recurrence cannot be determined by this clinical approach. Further clinical and basic science investigations should be pursued to address these issues.

CONCLUSIONS

Low telomere DNA content measured in the prostate needle biopsy tissue predicts early likelihood of post-prostatectomy PSA recurrence independent of pre-operative PSA and Gleason sum in a retrospective analysis, particularly in men with low to intermediate grade disease. This study indicates the potential clinical use of telomere DNA content in prostatic biopsies as an independent prognostic marker for early PSA recurrence after surgical treatment.

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LEGENDS

Figure 1. Kaplan-Meier survival analysis with respect to PSA recurrence-free survival for all subjects (n=103) for **A.** groups determined by a telomere DNA content (TC) cutoff of 0.30 and **B.** groups determined by a highest pre-operative PSA cutoff of 10 ng/mL. Kaplan-Meier survival analysis with respect to PSA recurrence-free survival for subjects with Gleason sum ≤7 (n=84) for **C.** groups determined by a telomere DNA content (TC) cutoff of 0.30 **D.** groups determined by a highest pre-operative PSA cutoff of 10 ng/mL. Tick marks indicate censored events.

Table 1. Characteristics of men undergoing radical prostatectomy included in the study population.

	All Study Subjects *n*=103			**Subjects with Gleason Sum ≤7** *n*=84		
	No Recurrence	**Recurrence**	***p*=**	**No Recurrence**	**Recurrence**	***p*=**
***n*=**	58 (56.3%)	45 (43.7%)		56 (66.7%)	28 (33.3%)	
Age at Diagnosis						
Mean (years)	62.7	64.1	0.24	62.9	63.3	0.75
Range	48-74	51-74		48-74	53-74	
Follow-Up Time						
Mean (months)	111	118	0.39	111	130	0.013
Range	1-163	28-165		1-163	88-165	
Time to PSA Recurrence						
Mean (months)	-	27		-	35	
Range	-	0-115		-	0-115	
Biopsy Telomere DNA Content						
Mean	1.17	0.94	0.17	1.21	0.82	0.035
Range	0.10-3.19	0.07-3.31		0.10-3.19	0.07-2.72	
Pre-Operative PSA (ng/mL)[†]						
Mean	7.97	11.33	0.031	7.93	9.96	0.18
Range	0.5-29.1	0.08-52.5		0.5-29.1	0.08-24.6	
Biopsy Gleason Sum						
≤6	39 (67%)	23 (51%)	0.19	39 (70%)	16 (57%)	0.54
7	9 (16%)	9 (20%)		9 (16%)	6 (21%)	
≥8	5 (9%)	10 (22%)		3 (5%)	3 (11%)	
Undetermined[‡]	5 (9%)	3 (6%)		5 (9%)	3 (11%)	
Pathologic Gleason Sum						
≤6	27 (47%)	8 (18%)	<0.0001	27 (47%)	8 (18%)	0.26
7	29 (50%)	20 (44%)		29 (50%)	20 (44%)	
≥8	2 (3%)	17 (38%)		-	-	
Organ Confined Disease	43 (74%)	17 (38%)	0.00027	41 (73%)	12 (43%)	0.0088
Seminal Vesicle Invasion	2 (3%)	13 (29%)	0.00039	2 (4%)	5 (18%)	0.038
Surgical Margin Involvement	15 (26%)	31 (69%)	<0.0001	14 (25%)	19 (68%)	0.00029

[†] Three men were missing documented pre-operative PSA values.

[‡] Limited tumor tissue in the biopsy sample prohibited the pathologist from assigning a Gleason sum.

Table 2. Univariate Cox proportional hazards models with respect to time (months) to PSA recurrence for the study population.

	All Study Subjects			Subjects with Gleason Sum ≤7		
	Hazard Ratio	**95% CI**	***p*=**	**Hazard Ratio**	**95% CI**	***p*=**
Biopsy Telomere DNA Content						
≥0.30	1.00			1.00		
<0.30	2.31	1.22-4.34	0.0097	4.11	1.92-8.81	0.00027
Pre-Operative PSA (ng/mL)						
<4.0	1.00			1.00		
4.0 – 10.0	1.97	0.67-5.81	0.22	1.49	0.48-4.62	0.49
≥10.0	3.47	1.18-10.17	0.023	2.05	0.64-6.56	0.22
Biopsy Gleason Sum						
≤6	1.00			1.00		
7	1.45	0.67-3.13	0.35	1.52	0.60-3.89	0.38
≥8	2.63	1.24-5.55	0.011	1.95	0.57-6.72	0.29
Pathologic Gleason Sum						
≤6	1.00			1.00		
7	2.04	0.90-4.63	0.090	2.07	0.91-4.71	0.083
≥8	5.28	4.33-24.33	<0.0001	-	-	-
Organ Confined						
No	1.00			1.00		
Yes	0.33	0.18-0.60	0.00032	0.37	0.17-0.77	0.0085
Seminal Vesicle Invasion						
Absent	1.00			1.00		
Present	4.06	2.12-7.80	<0.0001	2.49	0.95-6.56	0.065
Surgical Margin Involvement						
Absent	1.00			1.00		
Present	4.06	2.15-7.68	<0.0001	4.76	2.14-10.6	0.00013

Table 3. Multivariate Cox proportional hazards model demonstrating low telomere DNA content (<0.3) predicts early likelihood for PSA recurrence adjusted for pre-operative variables for the study population.

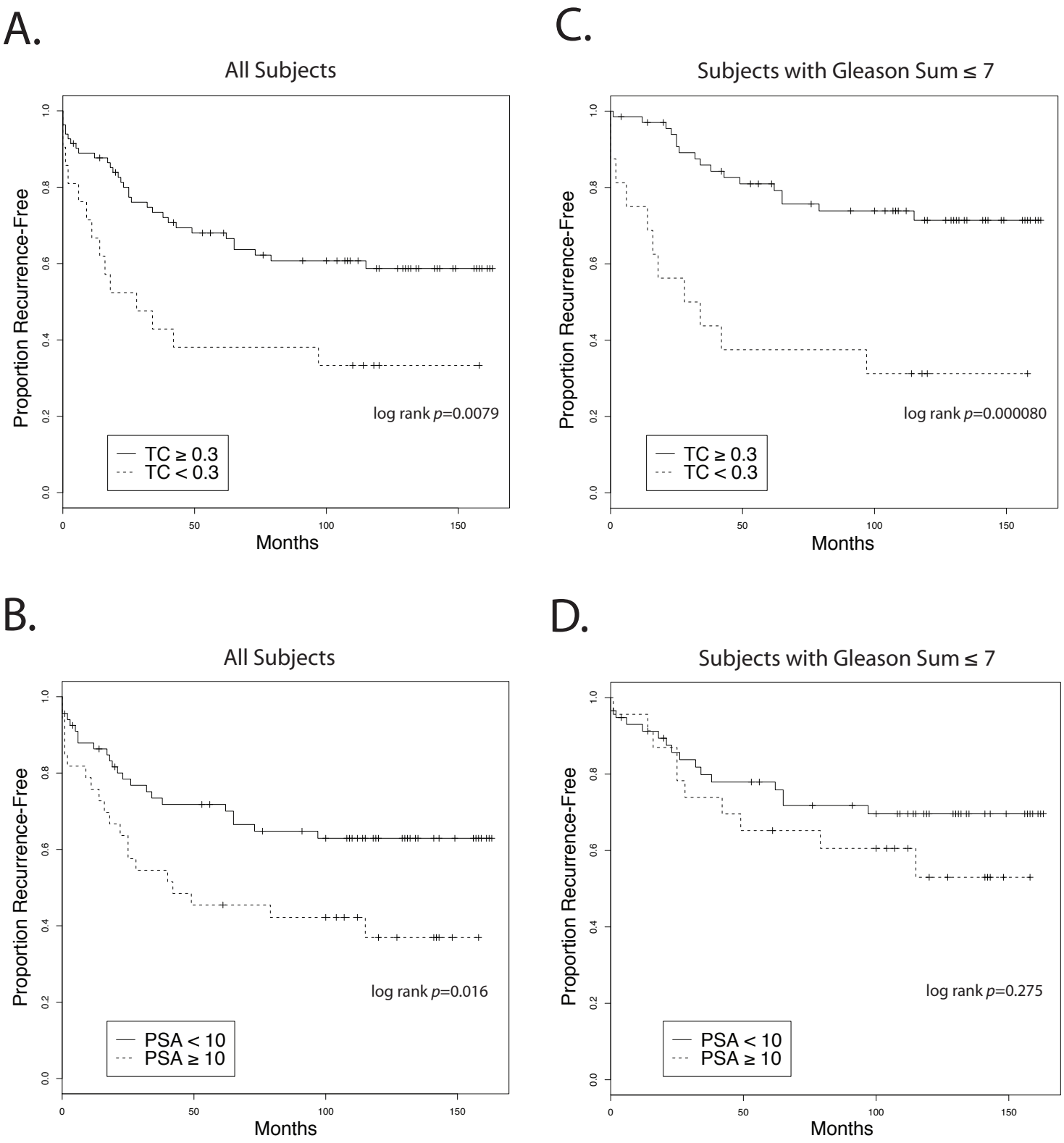
	All Study Subjects *n*=92†			**Subjects with Gleason Sum ≤7 *n*=73†**		
	Hazard Ratio	**95% CI**	***p*=**	**Hazard Ratio**	**95% CI**	***p*=**
Biopsy Telomere DNA Content						
≥0.30	1.00			1.00		
<0.30	2.56	1.23-5.32	0.012	8.31	3.26-21.5	<0.0001
Pre-Operative PSA (ng/mL)						
<4.0	1.00			1.00		
4.0 – 10.0	2.51	0.70-9.03	0.16	3.96	0.98-16.0	0.053
≥10.0	4.39	1.28-15.61	0.022	5.13	1.23-21.3	0.024
Biopsy Gleason Sum						
≤6	1.00			1.00		
7	1.59	0.72-3.51	0.25	2.27	0.84-6.17	0.11
≥8	1.69	0.73-3.88	0.22	0.75	0.20-2.82	0.67

† Subjects with incomplete or missing data were excluded from the analysis.

Table 4. Tests for association of low telomere DNA content (<0.3) with established prognostic markers for the study population.

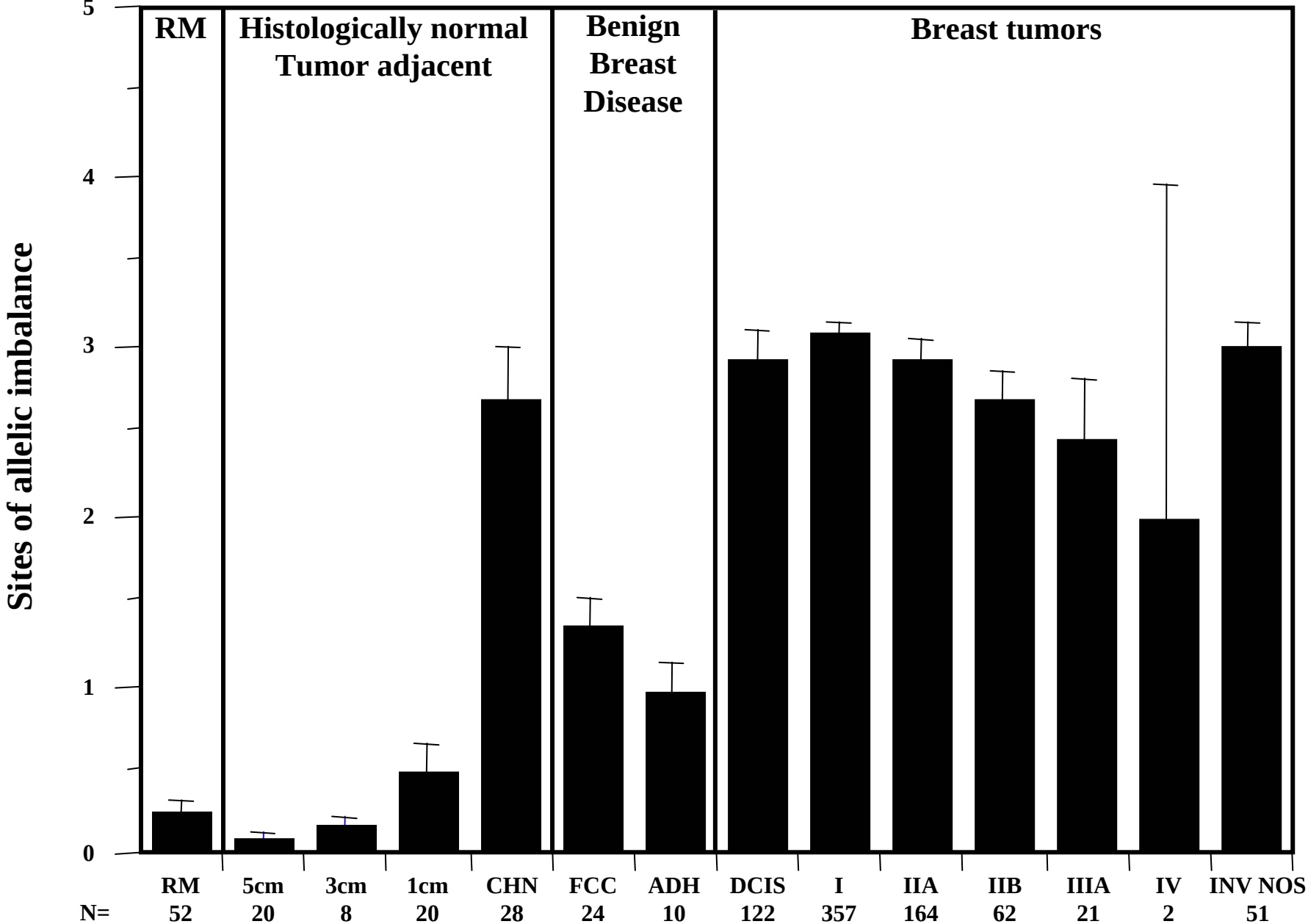
	***p*=**
Pre-Operative PSA (ng/mL)	
<4.0	0.51
4.0 – 10.0	
≥10.0	
Pre-Operative PSA >10 vs. ≤10	0.91
Pre-Operative PSA >4 vs. ≤4	0.45
Biopsy Gleason Sum	
≤6	0.48
7	
≥8	
Biopsy Gleason Sum >7 vs. ≤7	0.42
Biopsy Gleason Sum >6 vs. ≤6	0.92
Pathologic Gleason Sum	
≤6	0.76
7	
≥8	
Organ confined	0.18
Seminal Vesicle Invasion	0.76
Surgical Margin Involvement	0.58

Figure 1.


A.
All Subjects
Proportion Recurrence-Free
Months
log rank p=0.0079
TC ≥ 0.3
TC < 0.3
C.
Subjects with Gleason Sum ≤ 7
Proportion Recurrence-Free
Months
log rank p=0.000080
TC ≥ 0.3
TC < 0.3
B.
All Subjects
Proportion Recurrence-Free
Months
log rank p=0.016
PSA < 10
PSA ≥ 10
D.
Subjects with Gleason Sum ≤ 7
Proportion Recurrence-Free
Months
log rank p=0.275
PSA < 10
PSA ≥ 10

Transcript	Fold Increase TAHN-1	N	Fold Increase TAHN-5	N	Fold Increase RM	N	BRCA Related
COLLAGEN ALPHA 1(I) CHAIN PRECURSOR	16.16	6	4.24	5	ND	5	**Yes**
COLLAGEN ALPHA 1(III) CHAIN PRECURSOR	12.80	6	2.24	5	1.82	5	**Yes**
COLLAGEN ALPHA 2(I) CHAIN PRECURSOR	9.12	6	1.62	5	0.99	5	**Yes**
RASGAP-ACTIVATING-LIKE PROTEIN 2	6.44	6	2.44	5	ND	5	**Yes**
SCAN DOMAIN-CONTAINING PROTEIN 2	6.32	6	3.01	5	7.27	5	No
G25K GTP-BINDING PROTEIN, (CDC42 HOMOLOG)	6.24	6	2.48	5	7.78	5	**Yes**
FUSE BINDING PROTEIN 3	6.11	6	2.56	5	4.79	5	**Yes**
EPSIN 2 ISOFORM B; EPS15 BINDING PROTEIN	6.01	6	3.22	5	6.60	5	No
OK/SW-CL.87	5.82	6	2.88	5	6.42	5	No
CONNECTIVE TISSUE GROWTH FACTOR PRECURSOR	5.49	6	6.67	6	3.46	5	**Yes**
PROTO-ONCOGENE PROTEIN C-FOS	5.47	6	3.36	5	ND	5	**Yes**
EARLY GROWTH RESPONSE PROTEIN 1 (EGR-1)	5.34	6	4.24	6	12.39	5	**Yes**
CYTOCHROME P450 3A43 (EC 1.14.14.1)	5.32	6	3.00	5	6.91	5	No
SEX COMB ON MIDLEG-LIKE 2	5.29	6	3.86	5	5.25	5	No
NOVEL PHOSPHOGLUCOMUTASE LIKE PROTEIN	5.17	5	2.44	5	ND	5	No
ATAXIN-7	5.05	6	2.79	5	ND	5	No
LINE-1 REVERSE TRANSCRIPTASE HOMOLOG	4.92	6	3.11	5	5.60	5	No
CYR61 INSULIN-LIKE GROWTH FACTOR-BINDING PROTEIN	4.84	6	4.25	5	6.26	5	**Yes**
PROTON MYO-INOSITOL CO-TRANSPORTER (HMIT)	4.80	6	2.37	6	8.23	5	No
MHC CLASS I POLYPEPTIDE-RELATED SEQUENCE B	4.77	6	2.55	6	5.97	5	No
ZINC FINGER PROTEIN 36	4.68	6	2.42	5	ND	5	No
FOCAL ADHESION KINASE 1	4.66	6	3.46	6	5.90	5	**Yes**

Table 1. Known Genes Differentially Regulated Selectively and Reproducibly in TAHN-1 Tissues. Fold increase is calculated as the ratio of median expression in TAHN-1, TAHN-5, or RM tissues relative to 10 pooled RNAs from normal breast tissues. N is the number of tissue specimens that had even pixel intensities (i.e. linear regression ratio > 0.6) and a signal to noise ratio > 3.0. A gene was defined as breast cancer related if it was contained in the title of one or more peer-reviewed papers identified in a PubMed search using the gene's name and "breast cancer" as search terms. ND; not detectable.



Distribution of Allelic Imbalance (AI) in Normal and Abnormal Breast Tissues. The average number of sites of imbalanced loci per specimen is shown on the y-axis. The types of tissues that were examined are indicated on the x-axis. Legend: reduction mammoplasty, RM; tumor adjacent, histologically normal tissue from sites 5 cm from the tumor margin, TAHN-5; breast tissue with fibrocystic changes, FCC; atypical ductal hyperplasia, ADH; TNM Stages 0 (DCIS), I, IIA, IIB and IIIA. The number of tissues analyzed is indicated (N).

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MINI REVIEW

Telomeres: Prognostic markers for solid tumors

Marco Bisoffi, Christopher M. Heaphy and Jeffrey K. Griffith*

Department of Biochemistry and Molecular Biology, University of New Mexico School of Medicine, Albuquerque, NM, USA

Solid tumors continue to affect millions of people worldwide. Increasingly sophisticated diagnostic tools contribute to the high incidence rates for some tumor types, and treatment options continue to expand. However, the progression of solid tumors represents a challenge for the appropriate treatment of individual patients because of the relative inaccuracy of current prognostic markers, including the widely used Tumor-Nodes-Metastasis (TNM) staging system, to predict the course of disease. As a result, both over- and undertreatment are clinical realities in the management of patients diagnosed with solid tumors. Therefore, population-based screening programs that increase the overall cancer incidence rates are controversial, as they may do little to improve the patient's quality of life. Consequently, there is a strong need to develop novel and independent markers of prognosis. In this context, we review the use of telomeres as prognostic markers for solid tumors, including cancers from lung, breast, prostate, colon, brain and head and neck. Telomeric sequences, the repetitive DNA at the end of human chromosomes, are mediators of genomic stability and can undergo length alterations during tumor initiation and progression. In a number of studies reviewed here, these alterations, measured as telomere attrition and elongation, have been shown either to be associated with clinical markers of disease progression or to be independent markers of cancer prognosis. We conclude from these studies that careful assessment of telomere length or its proxies, such as telomere DNA content, will be part of novel risk assessment and prognostic modalities for patients with solid tumors.

Key words: prognosis; solid tumors; telomeres; biomarker

The epidemiology of solid tumors in the 21st century is characterized by overall decreasing mortalities with concomitant increasing incidences in many types of cancer.[1,2] This unique situation is multifactorial and stems from a combination of prolonged life expectancies, more sophisticated screening modalities, increased awareness of cancer among the population, and a rise in the repertoire and efficacy of therapeutic options.[3–5] The latter can lead to the cure of the cancer, but often does not eliminate the risk of subsequent recurrences. Unfortunately, most therapies are accompanied by side effects and complications that decrease the quality of life. These can be caused by the relative lack of specificity of many therapeutic regimens, such as in the case of chemotherapeutic agents,[4,5] or to the unwanted effects of physiologically active compounds on the intrinsic endocrine system, such as in the case of hormonal therapies.[6,7] These complications must be ultimately included into the clinical decision making, along with the age of the patient, personal preferences and of course the risk assessment performed at or shortly after diagnosis. Risk assessment is based on clinical or pathological staging, and aims largely at identifying patients at higher risk of cancer progression and recurrence who would benefit from more aggressive treatment. The latter also implies that patients with indolent disease would be spared overtreatment with regimens that cause unnecessary side effects, added health risks, expenses and a reduced quality of life without real benefits. In this context, the choice of treatment modalities often represents a dilemma for the physician and the patient.[8,9]

Taken together, these important considerations converge into a strong need for reliable prognostic and predictive biomarkers that are able, with high specificity and sensitivity, to correctly identify patients who would or would not benefit from specific therapeutic intervention, as well as to predict the response of their tumor to a specific line of therapy.[8–11] This review summarizes the use of telomere length or its proxies, such as telomere content, as potential prognostic markers in solid tumors, a possibility that has been raised in the last decade by a number of investigators.[12–24] A corresponding comprehensive discussion of these promising developments has not yet been offered, in spite of the growing body of knowledge. Excluded from this discussion are numerous reports on the prognostic value of telomeres in hematological malignancies, a research area that, in contrast to solid tumors, has been addressed in more depth and previously reviewed.[25] Also excluded from this review is the use of telomerase, the enzyme implicated in telomere maintenance, as a biomarker for diagnosis or prognosis, a research area that also has been previously reviewed.[26]

Biomarker discovery in solid tumors—A continuous need

Ideal biomarkers for solid tumors would serve one or more important functions in the initial risk assessment and subsequent clinical management of the corresponding patient. These include providing a basis for choice of initial treatment, prediction of survival, stratification of patients into clinical trials, prediction and monitoring of response to therapy, accurate communication among health care providers and uniform reporting of outcomes.[8–11,27] The Tumor-Nodes-Metastasis (TNM) staging system has been used since the 1950s, and meets most of the criteria outlined earlier.[10,11,27] Using this system, the patient is stratified into a specific stage. For example, the 5th edition of the American Joint Cancer Committee describes 4 main stages for breast and prostate cancer patients (I-IV) with cancer-specific substages based, for example, on the lymph nodal status and extracapsular extension.[10,11] While this system has proven, and continues, to be helpful in the risk assessment and prognosis of cancer patients, there are areas where the TNM staging system falls short. For example, it does not accommodate well the increasingly observed stage compression for breast and prostate cancer, i.e., the migration towards earlier stages (I and II) because of increasing patient screening and awareness.[3,10] Furthermore, the TNM staging system cannot be extended with additional biomarkers, such as markers of cellular proliferation (Ki67), cell cycle (cyclins) or signal transducers (Her2); in fact, their addition would complicate and undermine its relative simplicity.[10,27] These points emphasize the need to change the basis for cancer prognosis from one that builds on temporal determinism (TNM at diagnosis) to one that builds on biological determinism, which includes the molecular characteristics of a tumor and their change over time.[27]

Telomeres and genomic instability

Genomic instability is a critical factor in the initiation and progression of human cancers.[28] One mechanism that underlies genomic instability is loss of telomere function.[29] Telomeres are nucleoprotein complexes located at the extreme ends of eukaryotic chromosomes. Telomeres in human somatic cells are comprised of

***Correspondence to:** Department of Biochemistry and Molecular Biology, University of New Mexico School of Medicine, MSC08 4670, 1 University of New Mexico, Albuquerque, New Mexico 87131-0001, USA.
Fax: 1+505-272-6587. E-mail: jkgriffith@salud.unm.edu
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the hexanucleotide DNA sequence, TTAGGG, that is repeated 1,000 to 2,000 times.[30] Numerous telomere binding proteins, including the telomere repeat binding factors 1 and 2 (TRF1, TRF2), TRF1-interacting nuclear protein 2 (TIN2), tankyrase and protection of telomeres 1 (Pot1) are associated with these repeat regions and are important for telomere maintenance.[31] Telomeres stabilize chromosome ends and prevent them from undergoing degradation and recombination. With increasing cell divisions, telomeres shorten due to incomplete replication of the lagging strand during DNA synthesis. Under normal physiological conditions, cell cycle check points such as pRB and p53 control further cellular activities by activating genetic programs of cell cycle arrest, differentiation or senescence. Also, when telomeres become critically shortened and compromise genomic stability, chromosome ends activate DNA damage response pathways that can induce apoptosis.[32] In cancer, these mechanisms are often inactivated, and telomeres can become dysfunctional by several mechanisms, such as loss or alterations of telomere-binding proteins involved in telomere maintenance, and DNA damage due, for example, to oxidative stress. Total loss of telomeric DNA can promote the formation of end-to-end chromosome fusions which lack TTAGGG repeats at the fusion point. In contrast, when telomeres become dysfunctional through the loss of telomere binding proteins, in the absence of telomeric DNA erosion, end-to-end fusions are also formed, but TTAGGG repeats at the fusion point are observed.[29,31,33] Telomere loss may be compensated for by the recombination based alternative lengthening of telomeres (ALT) pathway[34] or, as seen in the majority of human cancers, by the enzyme telomerase.[35]

Telomeres in tumors are frequently altered, resulting in both telomere attrition and elongation, as compared to matched adjacent normal tissues.[36] Extensive alteration of telomeric DNA results in complex types of genomic abnormalities, including loss of heterozygosity, gene truncation and aneuploidy.[29,37] The cause-and-effect relation between dysfunctional telomeres and genomic instability implies that dysfunctional telomeres are also associated with complex and largely unknown altered gene expression. The latter is a primary source of phenotypic variability, which in turn drives the development of cell clones displaying progressively malignant traits, such as the potential for invasion and metastasis.[31,38] Since genomic instability alters gene expression, tumors with excessive telomere alterations are therefore likely to possess the most extensive phenotypic variability and have the greatest probability of containing cells capable of invasion, extravasation and metastasis, *i.e*., an aggressive tumor phenotype. For these reasons, numerous investigators have hypothesized that altered telomere length could predispose cells to possess the properties necessary to metastasize and cause recurrent disease, and thereby be a predictor of clinical outcome.[12–24] This concept is shown schematically in Figure 1.

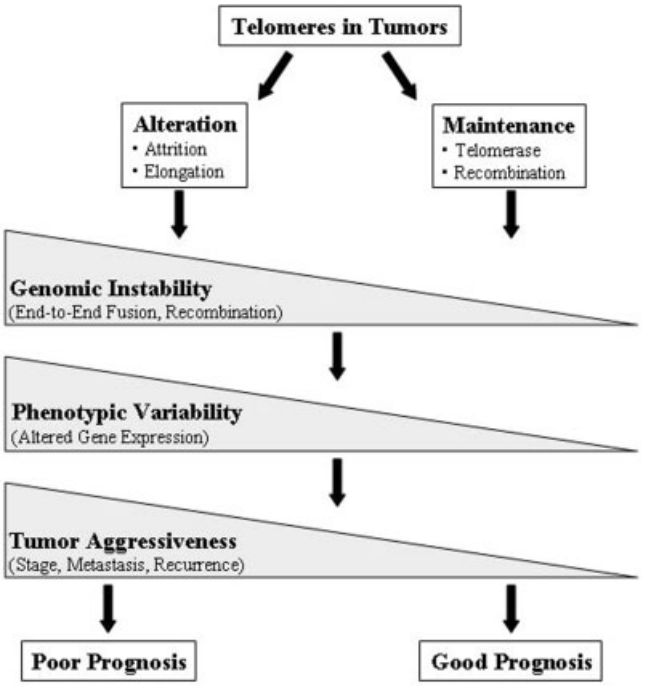

FIGURE 1 – Schematic representation of the cause-and-effect relationship between dysfunctional telomeres, genomic instability, phenotypic variability, tumor aggressiveness, and as a consequence, clinical outcome (poor/good prognosis). The triangles represent decreasing genomic instability, phenotypic variability and tumor aggressiveness from left to right.

Methods of telomere measurement

Several methods have been developed to measure telomere length, or its proxies, and have been recently reviewed by Lin and Yan[39] and by Baird.[40] These include the well-established Southern blot method, the telomere DNA content (TC) titration assay[41,42] and telomere fluorescence *in situ* hybridization (FISH).[40,43] All of the studies reviewed here have utilized the 2 former methodologies, ostensibly because of their ease of quantification or high-throughput capability. Therefore, these are briefly explained here; the reader is referred to Refs. 39, 40 and 43 for an explanation of FISH. Telomere length measurement using the Southern blot technique has been the most widely used method for assessing the length of telomeres in cells and tissues. Briefly, genomic DNA is digested with frequently cutting restriction endonucleases, such as *Hinf*I and *Rsa*I, to degrade nontelomeric sequences. The nonfragmented DNA containing the telomeric sequences, or terminal restriction fragments (TRFs) that are insensitive to restriction endonucleases, are then separated by gel electrophoresis and transferred to membranes suited for nucleic acid immobilization. Hybridization with a telomere-specific probe (*e.g.,* radioactively labeled) is performed and telomere length is usually assessed by densitometric analysis of the highest peak TRF signal after exposure of the blot to light-sensitive film or to a phosphorimager.[39,40] However, this well-established methodology harbors several inherent disadvantages. For example, Southern blots require relatively large amounts (*i.e*. micrograms) of originally unfragmented genomic DNA, which often precludes measurements of telomeric DNA in archival or biopsy tissues, where DNA can be degraded or scarce. In addition, Southern blot radiographs often display multiple peaks, leading to a high variability in interpretation.[39,40]

A method for assessing the total content of telomere DNA sequences (TC), a surrogate for telomere length, in genomic DNA was recently reported.[41,42] Briefly, genomic DNA is immobilized on membranes in a slot blot format and titrated by hybridization with a telomere-specific probe, either normalized to centromeric DNA[41] or to total genomic DNA in the same sample.[42] This methodology holds multiple advantages over the Southern blot technology: TC is independent of DNA fragmentation, DNA requirements are in the lower nanogram range, i.e. ~5 ng (equivalent of $<$1,000 cells) and measurements can be performed with DNA isolated from fresh, frozen and paraffin-embedded tissues up to 20 years old. Additionally, TC is directly proportional to mean telomere length and its interpretation is not affected by telomeric sequences outside the telomere.[42]

Regardless of the methodology used, the following discussion shows that telomeres can be used as clinical biomarkers in the prognosis of solid tumors, as reported in numerous studies.[12–24] These studies, and their major findings, are summarized in Table I and further discussed here in detail, according to the type of solid tumors analyzed.

Breast cancer

It has been estimated that worldwide over one million women are diagnosed annually with breast cancer, and over 200,000

TABLE I – SUMMARY OF FINDINGS FOR THE USE OF TELOMERES AS PROGNOSTIC MARKERS IN DIFFERENT TUMOR TYPES

Tumor type	*N*	Significant findings	Reference
Breast	41	TRF was reduced in Grade 3 tumors and in patients without lymph node metastases	Odagiri *et al.*[12]
	49	Reduced TC was correlated with aneuploidy and lymph node metastasis	Griffith *et al.*[13]
	140	Reduced TC was associated with tumor size, nodal involvement, TNM stage, 5-year overall survival and 5-year disease-free survival. Using multivariate Cox proportional hazards analysis, low TC was found to be a prognostic factor independent of age at diagnosis and TNM stage (RH = 4.43)	Fordyce *et al.*[16]
Prostate	18	Reduced TC correlated with death and disease recurrence	Donaldson *et al.*[14]
	77	Reduced TC predicted time to prostate cancer recurrence when controlling for age at diagnosis, Gleason sum score and pelvic node involvement (RH = 5.02)	Fordyce *et al.*[15]
Lung	46	Reduced and increased TRF was observed, but a clear association with patient prognosis was not reported	Shirotani *et al.*[22]
	72	Patients with altered (longer and shorter than normal) TRF had shorter survival durations and in a multivariate analysis; alteration in TRF was second to pathological stage in predicting the duration of patient survival	Hirashima *et al.*[23]
Colorectal	57	Increased TRF correlated with higher tumor stage, decreased overall survival, and in a multivariate analysis, TRF was an independent prognostic factor	Gertler *et al.*[18]
	91	Using multivariate Cox proportional hazards analysis, with a median follow-up time of 44 months, increased TRF was found to be a prognostic factor independent of tumor stage (RH = 6.48)	Garcia-Aranda *et al.*[19]
Head and neck	110	Patients with increased TRF in tumor tissues had decreased disease-free survival	Patel *et al.*[20]
Neuroblastoma	55	Reduced TRF was correlated with advanced stages of tumor development, poor prognosis, and increased S-phase fractions in tumor cells	Hiyama *et al.*[21]
Glioblastoma multiforme	77	The ''ALT'' phenotype, representative of elongated telomeres, was associated with a better prognosis	Hakin-Smith *et al.*[24]

women die from this disease.[44] The first report describing the use of telomere length as a possible prognostic marker in breast carcinoma was published by a group led by Odagiri *et al.*[12] In this study, Southern blot analysis was used to determine the telomere length in a cohort of 41 patients diagnosed with breast cancer and with a median follow-up time of 62.5 months. Additionally, tumor adjacent normal breast specimens were available for 22 of the patients. These authors found that telomere length was significantly reduced in the tumor tissues (8.1 $\pm$ 0.6 kb) compared with that of the adjacent normal (9.7 $\pm$ 0.5 kb) breast tissues in 18 of 22 patients ($p > 0.05$). Within the tumor specimens, 5/7 specimens with the shortest telomere lengths were Grade 3 tumors, whereas longer telomeres were seen in specimens with histologic Grades 1 or 2. However, no correlations were observed among telomere length and TNM stage, tumor size, estrogen receptor status, progesterone receptor status, age or disease-free survival. Interestingly, telomere length in the tumor adjacent normal tissues was also significantly shorter than telomere length in placental DNA (13.7 $\pm$ 0.4 kb). The latter is in agreement with studies in breast and prostate cancers, which show that telomere attrition takes place in histologically normal tissue adjacent to the corresponding tumors, and thus may represent an early event in the carcinogenic process.[15,45] At the time of their report, the authors hypothesized that telomere alterations may initiate and promote the development of malignancy.[12] In this context, Meeker *et al*. recently observed that telomere length abnormalities represent early and prevalent genetic alterations in the multistep process of malignant transformation in several types of cancer, including breast cancer.[46]

Two reports on telomeres measured as telomere DNA content, TC, one of the proxies of telomere length,[41,42] collectively indicate the prognostic potential of telomeres in breast cancer patients.[13,16] The first study, reported by Griffith *et al*., included 49 frozen and paraffin-embedded invasive human breast carcinomas. Tumors were divided into 3 groups (I–III) based on TC values.[13] All 16 tumors in the group with the least TC (Group I) were aneuploid, as compared to 9/17 tumors in the group with the highest TC (Group III). Statistical analysis of this association between TC and aneuploidy revealed a significant relationship between increased aneuploidy and reduced TC ($p < 0.002$). Furthermore, 12/14 tumors in Group I produced metastatic disease compared to 8/15 tumors in Group III. Statistical analysis of this distribution showed a significant association between metastatic disease, mostly nodal involvement, and reduced TC ($p < 0.05$). Finally, and in agreement with the previous study,[12] no association between TC and patient age, tumor size, grade, stage or fraction of cells in S-phase was observed.[13]

A more recent study reported measurements of TC in 2 independent sets of breast tumors containing a total of 140 samples.[16] This study by Fordyce *et al*. revealed that telomere alteration included both telomere attrition and elongation. In fact, only 50% of all tumors had TC values in the normal range, as defined by TC of 70 normal tissues of different types. Contrary to the previous 2 studies, TC in this larger cohort was associated with tumor size ($p = 0.02$), TNM stage ($p = 0.004$), 5-year overall survival ($p = 0.0001$) and 5-year disease-free survival ($p = 0.0004$). Reduced TC was also associated with nodal involvement ($p < 0.0001$), which is in contrast to the study by Odagiri *et al*. who found that decreased telomere lengths were observed in patients without lymph node metastases when compared to those with lymph node metastases ($p > 0.05$).[12] Finally, a multivariable Cox model to control for age at diagnosis and TNM stage as predictors of breast cancer-free survival showed that low TC conferred an adjusted relative hazard (RH) of 4.43 (95% CI 1.4–13.6, $p = 0.009$). Receiver operating characteristic curves using thresholds defined by the TC distribution in normal tissues predicted 5-year breast cancer-free survival with 50% sensitivity and 95% specificity, and predicted death due to breast cancer with 75% sensitivity and 70% specificity.[16]

In summary, multiple studies in breast cancer have shown that telomere attrition is associated with parameters of increased risk and poor outcome, and can thus be used as a prognostic factor to predict the course of disease. Overall, these studies contain no contradictory findings, although the reports may differ in the types of associations between telomere length and parameters of staging and risk assessment. The latter may either indicate inherent and unknown differences in the patient populations under investigation, or a limited number of specimens, which indicates the need for larger studies.

Prostate cancer

Prostate cancer is the male gender-specific counterpart of breast cancer with regard to worldwide prevalence and incidence.[44] Recent investigations suggest that reduced telomere length, measured as TC, is associated with poor clinical outcome and markers of disease progression in prostate cancer.[14,15] The first reported study represented an retrospective investigation of the relationship between clinical outcomes in patients with organ-confined prostate adenocarcinoma and TC.[14] In this study, an archival case-controlled cohort, composed of 18 men diagnosed with prostatic adenocarcinoma and treated with prostatectomy prior to 1993 was selected so that ~50% died within 10 years of diagnosis and 50% survived 10 years or longer. Association analysis for TC and survival and TC and biochemical recurrence (PSA levels of >2.5 ng/ml) revealed that reduced TC correlated with death ($p < 0.0001$) and disease recurrence ($p < 0.0001$). Also, there was no apparent association between TC and patients' ages at diagnosis, nodal statuses, pathological grades or Gleason sum scores.[14]

This finding was recently extended in a retrospective population-based study comprising 77 men who underwent prostatectomy between 1982 and 1995.[15] In this cohort, TC was determined in the tumor as well as in the tumor adjacent histologically normal prostate tissues. TC was a predictor of time to prostate cancer recurrence when controlling for age at diagnosis, Gleason sum score and pelvic node involvement (RH = 5.02, 95% CI 1.40–17.96, $p = 0.0132$). Interestingly, TC in tumors was associated with TC in the corresponding tumor adjacent tissues ($R = 0.601$, $p < 0.0001$). Median TC in men with cancer recurrence within 6 years was ~50% of TC in men who remained disease-free in tumor ($p = 0.012$), and surprisingly, also in tumor adjacent histologically normal tissues ($p = 0.024$). As mentioned earlier, the latter supports the concept, shown previously by us and others,[12,15,45] that telomere attrition takes place in histologically normal tissue adjacent to the corresponding tumors, and may thus represent an early event in tumor formation. This phenomenon may reflect ''field cancerization,'' a concept that was introduced to explain the occurrence of genetic alterations in histologically normal tissues adjacent to tumors.[47]

Lung cancer

Lung cancer is the most common malignancy and the leading cause of mortality related to cancer in the world.[44] It is thus somewhat surprising that research on telomeres in lung cancer specimens is relatively rare. While telomerase expression and activity have been recognized as important contributors to the malignant phenotype in lung epithelial cells, and have been proposed to be of potential prognostic value,[48] reports on the use of telomeres to predict lung cancer progression are scarce. Using telomere length measurements by the TRF method, Shirotani et al. investigated the relationship between telomere length and various characteristics of tumor cells in 46 lung cancer specimens comprising 40 primary and 6 metastatic lesions.[22] Interestingly, and in partial accordance with recent studies in breast and prostate cancers,[15,16] these authors observed both elongation (2 cases) and reduction of telomere length (13 cases) in the 16 small cell carcinomas of the sample set. The 2 cases with telomere elongation were associated with a poor prognosis. Similarly, in the adenocarcinoma samples of this study, both telomere reduction and elongation were observed, but a clear association with patient prognosis was not reported.[22]

Hirashima *et al*. evaluated the prognostic significance of telomere length alteration in paired cancer and normal lung tissues obtained from 72 patients with histologically confirmed pathological stage I-IIIA nonsmall cell lung cancer (NSCLC).[23] TRF length (mean ± SD) in normal tissue was 6.2 ± 1.1 kb. These authors defined the upper and lower limits of a range of TRF length in normal tissue as 8.4 kb (mean + 2SD) and 4.0 kb (mean − 2SD), respectively. Defining telomere alteration as TRF values above or below these values, these authors determined that the 25 patients (34.7%) with alteration in TRF length (elongation and reduction) had significantly shorter survival durations than those of the others. In multivariate analysis, alteration in TRF length ($p = 0.0033$) was second to pathological stage ($p = 0.0004$) in predicting the duration of patient survival, indicating that telomeres in this type of cancer can be of potential prognostic value.[23]

Although not entirely consistent in the type of telomere alteration, *i.e*., attrition *vs*. elongation, and unclear on the underlying mechanisms, these studies in lung tissues indicate that telomere alterations are associated with parameters of clinical outcome in patients suffering from lung cancer.

Colorectal carcinoma

Although colorectal cancer is one of the best studied malignant diseases in terms of genetics and molecular prognostic factors, the true prognostic significance of all potential factors under investigation remains to be clarified.[49] The use of telomeres as prognostic factors in colorectal carcinoma has been addressed in several studies in the groups led by Siewert and coworkers[17,18] and by Iniesta and coworkers.[19] As with most previous studies, these authors measured telomere length by the Southern blot method. In a study encompassing 57 patients with completely resected colorectal carcinoma and a median follow-up time of 76 months, Gertler *et al*. first determined that cancer tissue had shorter telomeres than adjacent mucosa ($p < 0.001$), and that telomere length decreased with age only in noncancerous tissue ($R = 0.36$; $p < 0.01$).[18] Furthermore, these authors determined the association between telomere length and histopathological parameters and patient survival. Telomere length in cancer tissue was correlated with tumor stage, with significantly shorter telomeres in stage I compared to stage II-IV tumors ($p < 0.01$). Patients with ratios of telomere length in cancer to noncancer tissue greater than 0.90 had a significantly poorer overall survival compared with patients with smaller telomere length ratios ($p < 0.002$). In multivariate analysis, the telomere length ratio was an independent prognostic factor ($p < 0.03$). Interestingly, and in agreement with recent findings in prostate and breast tumors,[15,16] as well as some findings reported in lung cancer,[22] these authors found a subset of patients whose tumors showed elongated telomeres as compared to their normal noncancerous tissues, indicating that telomere maintenance mechanisms can be shifted towards telomere elongation in this type of cancer tissue.

Similar findings were reported by Garcia-Aranda *et al*. in a study encompassing 91 primary colorectal carcinomas and their associated normal control tissue samples.[19] Again, telomeres in cancer specimens were significantly shorter compared with telomeres in normal, adjacent tissues ($p = 0.02$). For the prognostic evaluation of telomere length, these authors dichotomized the patient cohort into samples with longer telomeres (*i.e*., the upper 2 quartiles above the mean in the tumor tissues) and samples with shorter telomeres (*i.e*., the lower 2 quartiles below the mean in the tumor tissues), and used a median follow-up time of 44 months. Using multivariate Cox proportional hazards analysis, telomere length in this cohort was found to be a prognostic factor independent of tumor stage, with an overall relative risk for elongated telomeres of 6.48 ($p = 0.04$).[19]

It is noteworthy that these studies on telomere length and their association with prognosis in colorectal carcinoma patients showed the opposite relationship, with longer telomeres indicating poor prognosis, as compared to studies in other cancer types[13–16,21] One

explanation for this discrepancy might lie in the differential regulation of telomerase expression in colorectal epithelial cells. In this context, normal colorectal epithelium has been shown to contain cells of possible stem cell origin that are telomerase-positive and presumably counteract telomere attrition due to physiologically high cell proliferation rates, and total cell loss due to physiological shedding in this specialized cell compartment.[50] It is thus possible that the regulation of existing telomerase activity may be affected in cells undergoing tumor initiation and result in elongated telomeres in the tumors.

Other types of cancer

Studies indicating the prognostic potential of telomeres have also been reported for head and neck cancer,[20] neuroblastomas[21] and glioblastomas.[24] Patel *et al*. studied telomere alterations by Southern blot analysis in tumor and adjacent normal tissues in 110 patients with head and neck cancer and 40 patients with precancerous and benign conditions.[20] Telomere lengths in this sample set were significantly lower in malignant tissues compared with the tumor adjacent normal tissues. In addition, while no significant correlation was observed between telomerase activation and clinicopathologic characteristics of the patients, 2-year disease-free survival analysis showed that patients with longer telomeres in malignant tissues had poor disease-free survival. The finding that elongated telomeres are associated with poor prognosis is in concert with that reported for colorectal carcinoma and lung,[17–19,23] and in contrast to other cancer types.[13–16,21] A possible explanation for these interesting discrepancies in head and neck cancer is the fact that telomerase activity was observed in over half of the adjacent normal tissues. As in the prior case of colorectal carcinoma, altered regulation of telomerase expression in cells undergoing transformation may explain the elongated telomeres in the tumors.[17–19,50]

A study by Hiyama *et al*. reported the reduction of telomere DNA repeats in 55 primary neuroblastomas as compared to ganglioneuromas and normal peripheral mononuclear cells.[21] It is interesting to note that these authors observed a wide range of telomere lengths in neuroblastomas (1.1 kb to >23 kb). Most importantly, the reduction of telomeric repeats within this sample set was significantly correlated with advanced stages of tumor development, poor prognosis and increased S-phase fractions in tumor cells.[21]

Finally, Hakin-Smith and colleagues determined telomere length, as well as the lengthening mechanism, either telomerase or ALT,[34] in 77 patients with glioblastoma multiforme, a brain tumor type for which no reliable prognostic markers exist.[24] Nineteen patients (25%) had tumors with the ALT phenotype, which is representative for longer telomeres. Median survival for patients with the ALT phenotype was 542 days (95% CI 114–970) compared with 247 days (95% CI 224–270) for glioblastoma multiforme tumors with normal telomeres ($p = 0.0003$). Cox regression analysis showed that this association was independent of age. These findings suggest that ALT, and by inference, elongated telomeres, are indicators of a favorable prognosis in patients with glioblastoma multiforme.[24] The latter finding is in agreement with observations made in breast tumors, where higher TC values, *i.e*., greater than that of the lowest tertile as defined in disease-free breast tissues, was associated with a better patient prognosis.[16]

Conclusion

The phenomenon of telomere alteration during tumorigenesis process and progression of solid tumors is well known and established at the molecular level.[29,37] The relationship between telomere maintenance, genomic instability and the resulting phenotypic variability that gives rise to cell clones that cause disease recurrence (Fig. 1) is well accepted.[31,38] It is only logical then to infer that alterations in telomere length could be used as a prognostic factor. Consequently, this review shows that the potential clinical use of telomere length information for the prognosis of solid tumors has been recognized and continues to be validated (Table I).[12–24] Also, some of these studies indicate not only an association of telomere length with prognosis, but also with parameters of staging and risk assessment. This suggests that even in cases where such an association was not observed, it may be detected in a larger sample size with more statistical power, emphasizing the need for extended, possibly multi-center studies. We argue that the phenomenon of bidirectional telomere dysfunction, including attrition or elongation, is subjected to the same overarching characteristic for almost all solid tumors, *i.e*. heterogeneity. The latter may increase with increasing genomic instability, affecting the molecular key players of telomere maintenance. At least two mechanisms of telomere maintenance, telomerase activity and the recombination-based ALT, may be more or less prevalent in different tissues undergoing tumor formation, leading to the observed differences.[34,35] Nevertheless, the studies reviewed here do indicate a potential tissue-specific pattern of telomere dysfunction that may reflect the underlying biology of telomere maintenance and its alteration over time in specific tissues. The latter is, for example, the case for normal colorectal epithelium that is known to contain telomerase-positive cells of possible stem cell origin.[50] Many questions remain to be answered, such as the exact definition of ''shortened'' or ''elongated'' telomeres. While the latter might be highly dependent on the type of tissue under investigation, several investigators have started to address this question by comparing telomeres in tumors to telomeres in a variety of normal disease-free human tissues.[12,16] Also, as indicated by several studies including our own,[12,15,45] a better understanding of the molecular events underlying telomere alterations in histologically normal tissues adjacent to tumors will probably open new research avenues into the identification of early diagnostic and prognostic markers.

It is clear that the reports reviewed here, although overall of a proof-of-principle character, clearly support the concept of using telomeres as independent biomarkers for prognosis.[12–24] We therefore conclude and predict that careful assessment of telomere length or its proxies, such as telomere DNA content or other methods to be developed in the future, will be used for solid tumor risk assessment, staging and prognosis. In addition, Hammond and Taube, as well as Weidner, propose in their marker discovery schemes that novel markers should be independent of previously discovered ones.[8,9] However, the possibility that telomere length, or its proxies, could represent combinatorial markers, for example, in more complex algorithms, should not be excluded.

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Telomere Content Correlates with Stage and Prognosis in Invasive Breast Cancer

CM Heaphy, CA Fordyce, M Bisoffi, JL Wyaco, NE Joste, A Mangalik, KB Baumgartner, RN Baumgartner, WC Hunt and JK Griffith, University of New Mexico School of Medicine, Albuquerque, NM,

Background: Telomeres are nucleoprotein complexes that protect chromosome ends from degradation and recombination. Critically shortened telomeres generate genomic instability, and it has been postulated that the extent of telomeric DNA loss is related to the degree of genomic instability within a tumor, and therefore may presage clinical outcome. The objectives of this investigation were to first define the range of telomere DNA contents (TC) in normal breast tissues and then evaluate the hypothesis that that TC in breast tumor tissue correlates with TNM staging and prognosis.

Methods: Slot blot assay was used to quantitate TC in 70 disease-free normal tissues from multiple organ sites, and two independent sets of breast tumors containing a total of 140 samples. Non-parametric Rank Sums Tests, Logistic regression and Cox proportional hazards models were used to evaluate the relationships between TC and tumor size, nodal involvement, TNM stage, 5-year survival and disease-free interval.

Results: TC in 95% of normal tissues was 75-143% of that in the placental DNA standard, whereas only 50% of tumors had TC values in this range. TC was associated with tumor size ($p=0.02$), nodal involvement ($p<0.0001$), TNM stage ($p=0.004$), 5-year overall survival ($p=0.0001$) and 5-year disease-free survival ($p=0.0004$). A multivariable Cox model was developed using age at diagnosis, TNM stage and TC as independent predictors of breast cancer-free survival. Relative to the high TC group (>123% of standard), low TC (<101% of standard) conferred an adjusted relative hazard of 4.43 (95% CI 1.4-13.6, $p=0.009$), independent of age at diagnosis, nodal involvement and TNM stage. Receiver operating characteristic curves using thresholds defined by the TC distribution in normal tissues predicted 5-year breast cancer-free survival with 50% sensitivity and 95% specificity, and predicted death due to breast cancer with 75% sensitivity and 70% specificity.

Conclusions: TC in breast cancer tissue is an independent predictor of clinical outcome and survival interval, and may discriminate by stage.

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Protease nexin-1 expression is altered in human breast cancer

Britny J Candia, William C Hines, Christopher M Heaphy, Jeffrey K Griffith and Robert A Orlando*

Address: Department of Biochemistry and Molecular Biology, University of NewMexico, School of Medicine, MSC08 4670, 1 University of New Mexico, Albuquerque, New Mexico, 87131, USA

Email: Britny J Candia - bcandia@unm.edu; William C Hines - Curthines@aol.com; Christopher M Heaphy - cheaphy@salud.unm.edu; Jeffrey K Griffith - jkgriffith@salud.unm.edu; Robert A Orlando* - rorlando@salud.unm.edu

* Corresponding author

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Abstract

Background: Urokinase-type Plasminogen Activator (uPA), a serine protease, plays a pivotal role in human breast cancer metastasis by mediating the degradation of extracellular matrix proteins and promoting cell motility. In more advanced breast cancers, uPA activity is significantly up regulated and serves as a prognostic indicator of poor patient outcome. Classically, regulation of uPA activity, especially in breast cancers, is thought to be mediated by Type 1 Plasminogen Activator Inhibitor (PAI-1). However, we have recently found that a lesser known natural inhibitor of uPA, Protease Nexin 1 (PN-1), is expressed in normal human mammary tissue. Based on this observation, we investigated if PN-1 is also expressed in human breast cancers where it may contribute to the regulation of uPA and participate in the development of a metastatic phenotype.

Results: Using quantitative real-time PCR analysis, we measured PN-1 mRNA expression in tissues obtained from 26 human breast tumor biopsies and compared these values with those obtained from 10 normal breast tissue samples. Since both PAI-1 and uPA expression levels are known to be elevated in metastatic breast cancer, we also measured their levels in our 26 tumor samples for direct comparison with PN-1 expression. We found that PN-1 expression was elevated over that found in normal mammary tissue; an increase of 1.5- to 3.5-fold in 21 of 26 human breast tumors examined. As anticipated, both PAI-1 and uPA mRNA levels were significantly higher in the majority of breast tumors; 19 of 26 tumors for PAI-1 and 22 of 26 tumors for uPA. A quantile box plot of these data demonstrates that the elevated PN-1 expression in breast tumor tissues directly correlates with the increased expression levels found for PAI-1 and uPA.

Conclusion: The fact that PN-1 expression is elevated in human breast cancer, and that its increased expression is directly correlated with increases measured for PAI-1 and uPA, suggests that PN-1 may contribute to the regulation of uPA-mediate tumor cell motility and metastatic spread.

Background

An important characteristic of highly invasive tumor cells is an elevated capacity to degrade the surrounding extracellular matrix (ECM). To achieve this elevated degradative capacity, tumor cells express a variety of proteases to digest ECM proteins that typically encapsulate growing,

benign tumors [1,2]. It is now well established that proteins of the plasminogen activation (PA) system are elevated in breast cancer and serve as the primary functional players in ECM degradation [3,4]. Expression of one member of the PA system, the serine protease urokinase (uPA), is significantly upregulated in tumor cells and catalyzes the conversion of extracellular plasminogen to plasmin [5]. Plasmin is a broad-spectrum protease that cleaves many ECM proteins, as well as activates certain matrix metalloproteinases [6]. This proteolytic cascade enables highly migratory tumor cells to efficiently degrade their surrounding matrices, exit the primary site of tumor growth and colonize distant secondary sites [7]. In addition to its protease activity which augments breast tumor cell motility, high expression levels of uPA is also a well-established prognostic indicator of poor patient outcome during the course of breast cancer [8,9].

Regulation of extracellular uPA activity is known to occur through the inhibitory properties of type I plasminogen activator inhibitor (PAI-1), a serine protease inhibitor (SERPIN) that is synthesized and secreted often by the same cells that secrete uPA [10]. Because of the close functional relationship between uPA proteolytic activity and PAI-1 inhibitory function, it is thought that a well-controlled balance of uPA and PAI-1 dictates the extent of cell motility. Protease Nexin-1 (PN-1), another member of the SERPIN family [11], is highly expressed by stromal cells [12] and a potent inhibitor of uPA [10,13]. Interestingly, although PN-1 activity has been extensively studied within the context of neural development, few studies have been reported examining its expression in cancerous tissues and its potential role in cancer progression. PN-1 is expressed by astrocytes and glial cells [14], as well as neuroblastoma cells [15] where it is thought to promote neuronal cell survival [16] and modulate neurite outgrowth [17]. In addition, PN-1 inhibits thrombin-stimulated cell division [18], migration of cerebrellar granular cells [19], and uPA-dependent ECM degradation [20]. Thus, based on findings in other cell types, we hypothesize that PN-1 may contribute to tumor cell motility in advanced stage breast cancer by playing a role in the regulation of uPA activity. To address this hypothesis, we examined the expression of PN-1 in advanced stage human breast cancer tissues to determine if its expression is altered when compared to normal mammary tissue and to directly compare its expression level to those of PAI-1 and uPA. To accomplish this goal, we used quantitative real-time reverse transcription-PCR (QRT-PCR) to measure PN-1, PAI-1 and uPA expression levels within a set of breast tumor and normal breast tissue samples.

Materials and methods

Breast tissue samples

Frozen breast tumor specimens from anonymous patients (n = 26) were obtained from the University of New Mexico Cancer Research and Treatment Center Solid Tumor Facility, Albuquerque, New Mexico. In 25 out of 26 cases, tumor grade, tumor size, lymph node status, and the fraction of cells in S phase (based on flow cytometry cell cycle analysis) were included within the clinical history provided with each specimen. Anonymous normal breast mRNA (n = 10) originating from female patients where cause of death was unrelated to cancer, were purchased from Ambion (Austin, TX). The normal, control samples were supplied as two equal pools by the company.

Cell culture

MCF-7 human mammary epithelial cells, were provided by Dr. Steven Abcouwer, Hershey Medical Center, Hershey, Pennsylvania. MDA-MB-231 metastatic human mammary epithelial cells were obtained from American Type Culture Collection (Rockland, MD). Both cell lines were propagated in Dulbecco modified Eagle's medium (DMEM, Life Technologies/Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum (Irvine Scientific, Santa Ana, CA), 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 0.01 mg/ml bovine insulin, and 100 U/ml penicillin G. Cells were cultured at 37°C with 5% CO_2 and passaged once a week.

Preparation of tissue sections and RNA isolation

Serial frozen sections of breast samples, 10 μm in width, were mounted on Colorfrost slides (VWR, West Chester, PA) and stored at -70°C. Specimens were stained with hematoxylin/eosin and examined by a board-certified surgical pathologist, who assigned a histopathologic grade to the tumor and analyzed the normal tissue. Total RNA from cultured cells and frozen tumor tissue was isolated using silica-based spin-column extraction kits (RNeasy/DNeasy mini kits, Qiagen, Valencia, CA) according to the manufacturer's protocol. Total RNA was treated with RNase-free DNase I (Ambion, Austin, TX) to eliminate contaminating DNA. RNA integrity was evaluated by agarose gel electrophoresis.

Quantitative real-time RT-PCR

cDNA was synthesized by random decamer-primed reverse transcription of RNA (1 μg) using a TaqMan® Reverse Transcription kit (Applied Biosystems, Foster City, CA) according to the manufacturer's standard protocol. Negative controls contained RNase-free water substituted for reverse transcriptase. The mRNA levels of PN-1, PAI-1, uPA and TATA-binding protein (TBP) were measured in breast specimens, the MCF-7 mammary epithelial cell line, and the MDA-MB-231 metastatic mammary epithelial cell line using the ABgene Absolute SYBR Green

QRT-PCR assay (Fisher Scientific, Hampton, NH). PN-1 primers were selected to amplify an 81 bp sequence spanning the intron located between exons 2 and 3. Primer sequences used for PN-1 were 5'-GAAGCAGCTCGCCATGGT-3' (forward), 5'-AGACGATGGCCTTGTTGATC-3' (reverse). TBP primer sequences used were 5'-CACGAACCACGGCACTGATT-3' (forward), 5'-TTTTCTTGCTGCCAGTCTGGAC-3' (reverse). Primer sequences used for PAI-1 were 5'-TGCTGGTGAATGCCCTCTACT-3' (forward), 5'-CGG TCA TTC CCA GGT TCT CTA-3' (reverse). uPA primer sequences used were 5'-CAC GCA AGG GGA GAT GAA-3' (forward), 5'-CA GCA TTT TGG TGG TGA CTT-3' (reverse) [21]. Final concentration of PN-1, PAI-1 and uPA primers used for amplification was 600 nmol/L forward, 600 nmol/L reverse; 600 nmol/L forward, 900 nmol/L reverse was used for TBP primers. Amplification of PN-1, PAI-1, uPA and TBP cDNA was performed using the MiniOpticon Real-Time PCR Detection System (Biorad, Hercules, CA). The cycling parameters used were as follows: 1 cycle, 95°C for 10 min; 50 cycles, 95°C for 15 sec and 60°C for 1 min; 1 cycle, 40°C for 3 min. The PN-1, PAI-1 and uPA mRNA levels were normalized to TBP mRNA levels using the Comparative C_T method and are reported in the figures as fold difference compared to levels found in normal mammary tissue. Melting curve analyses were performed for all amplifications to verify that only single products were generated from the reactions. Amplicons were sequenced to verify authentic PN-1. The cDNA for human PN-1 was obtained from the I.M.A.G.E. Consortium (ID: 4824856; Genbank: BC042628; Genbank: BC042628).

Results

Quantitation of PN-1 expression in human breast tumors

For QRT-PCR analysis, we designed primers to amplify an 81 bp sequence of PN-1 spanning the splice junction between exons 2 and 3. Spanning a splice junction ensures that amplified products are derived solely from mRNA and not from genomic DNA that might remain in our preparation. In order to test the specificity of these novel primers, we amplified the 81 bp PN-I sequence by straight RT-PCR using RNA purified from a normal human fibroblast cell line (HuFb) and compared the product to that obtained from amplification using the human PN-1 cDNA. We chose to use human fibroblasts since they synthesize and secrete active PN-1 at levels corresponding to ~1% of all secreted proteins [22,23]. As anticipated, we found that our newly designed primers amplified only the expected 81 bp sequence (data not shown).

To quantify PN-1 expression in human breast cancers, we obtained 26 samples of breast tumor tissue, purified RNA and generated cDNA from this material. The cDNA were then analyzed by quantitative PCR and the results were compared directly to quantitative PCR values obtained using cDNA generated from normal human mammary tissue. We found that PN-1 expression was elevated over that found in normal mammary tissue; an increase of 1.5- to 3.5-fold in 21 of 26 human breast tumors examined (Fig. 1A). Since both PAI-1 [24-26] and uPA [24,26] expression levels are known to be elevated in metastatic breast cancer, we measured their levels in our 26 tumor samples for direct comparison with PN-1 expression. As anticipated, both PAI-1 (Fig. 1B) and uPA (Fig. 1C) mRNA levels were significantly higher in the majority of breast tumors; 19 of 26 tumors for PAI-1 and 22 of 26 tumors for uPA. A quantile box plot of the data shown in Figure 1 permits a direct comparison of expression levels for PN-1, PAI-1 and uPA (Fig. 2). These data clearly show that PN-1 expression is elevated in the majority of human breast cancers examined and that this elevated expression directly correlates with the expected higher expression levels found for PAI-1 and uPA. Since the majority of our tumor samples represent advanced stage, grade 2 and 3 breast cancers, we are unable to determine at this time if PN-1 expression levels correlate with tumor grade, lymph node status or patient reoccurrence.

PN-1 expression in MCF-7 and MDA-MB-231 breast cancer cells

We plan to characterize the mechanism responsible for increased expression of PN-1 in breast cancer and determine its functional role in breast cancer metastasis. To accomplish this goal, we will require a cultured model system for accurate, controlled assessment of PN-1 promoter status, transcription factor requirements, and tumor cell invasive capacity. MCF-7 and MDA-MB-231 cells are well established cultured lines used extensively to study molecular details of breast cancer progression [27]. Hormone-responsive MCF-7 cells have a low invasive capacity and represent earlier stages of breast cancer, while hormone-independent MDA-MB-231 cells are highly invasive and represent more advanced stage breast cancer. To determine if these cell models will be useful for examining PN-1 function in breast cancer, we performed QRT-PCR analysis to identify if PN-1 expression is disregulated in a comparable manner to that seen in human breast cancer tissues. We found that MDA-MB-231 cells express 3.5-fold greater levels of PN-1 than MCF-7 cells (Fig. 3). Moreover, increased expression of PAI-1 and uPA were also found in MDA-MB-231 cells as compared to MCF-7.

Discussion

Advanced stage breast cancer is accompanied by a dramatic increase in metastatic potential of epithelial-derived tumor cells. The observed increase in tumor cell motility is aided by increased expression and activity of uPA [4]. For effective tumor cell migration, the proteolytic activity of uPA is thought to be balanced by the inhibitory activity

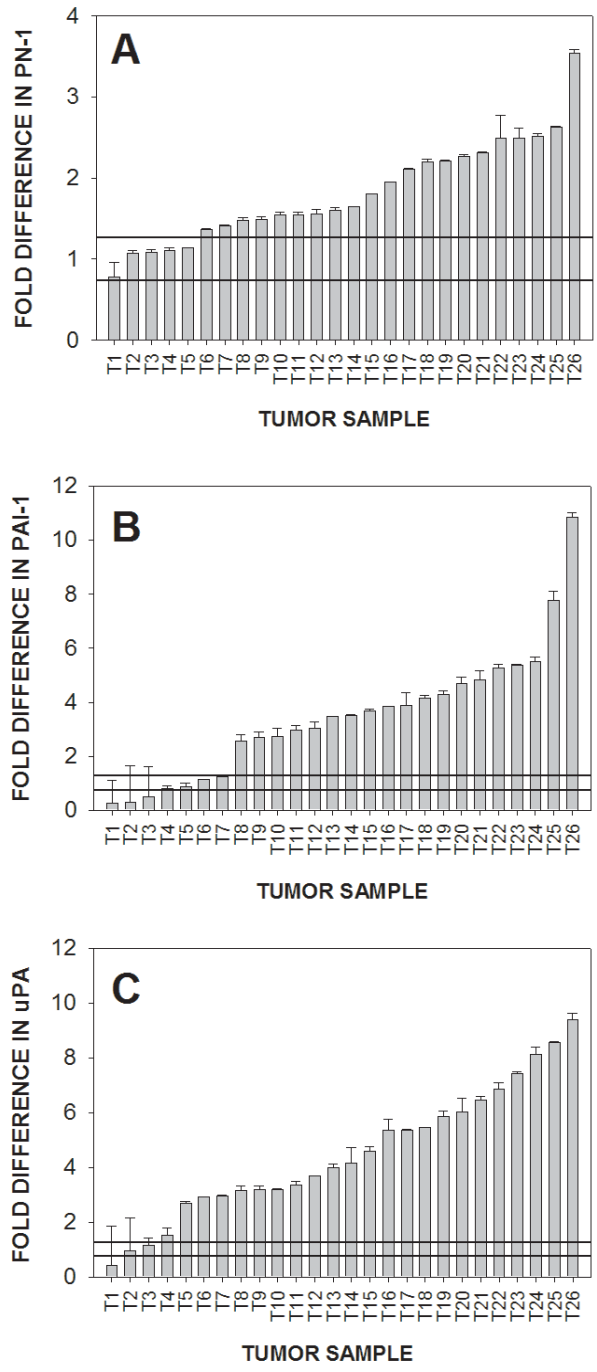

Figure 1

PN-1, PAI-1 and uPA mRNA expression in human breast tumor tissues and normal human mammary tissue. RNA was isolated from 26 breast tumors (T1–T26) and 10 normal breast samples. Normal samples were pooled into two equal groups (N1 and N2). mRNA levels for each gene were evaluated by QRT-PCR. Relative levels of PN-1, PAI-1 and uPA mRNA were normalized to TATA binding protein mRNA levels. Comparative C_T method was used to calculate fold difference of PN-1 (A), PAI-1 (B) and uPA (C) expression in breast tumor tissue as compared to levels measured in normal breast tissue. The mean value of the two pooled normal samples was calculated and assigned a value of one in order to determine relative fold change of expression within the tumor samples. The standard deviation of the normal samples was 0.263. The box represents one standard deviation of the mean values obtained from normal mammary tissues.

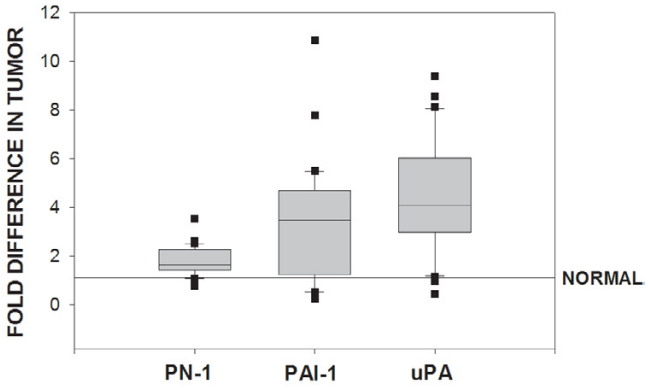

Figure 2
Comparison of PN-1, PAI-1 and uPA mRNA expression levels in 26 breast tumor samples. The box for each gene represents the interquartile range (25–75th percentile) and the line within this box is the median value. Bottom and top bars of the whisker indicate the 10th and 90th percentiles, respectively. Outlier values are indicated (closed squares).

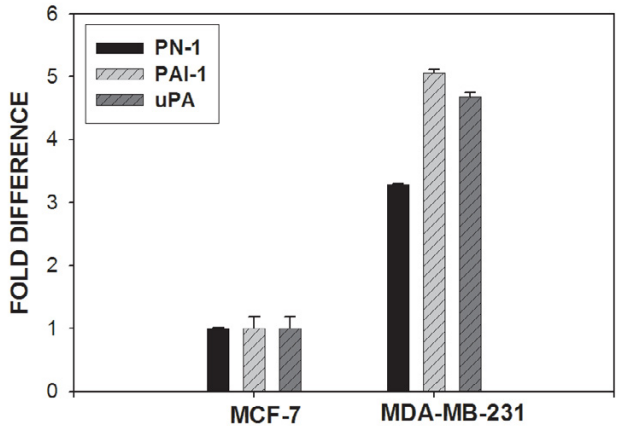

Figure 3
QRT-PCR analysis of PN-1, PAI-1 and uPA mRNA expression in MCF-7 and MDA-MB-231 breast cancer cell lines. PN-1, PAI-1 and uPA message levels were quantitated in MCF-7 and MDA-MB-231 cells by QRT-PCR. Expression levels for PN-1, PAI-1 and uPA were normalized to values obtained for TATA binding protein. C_T values for each gene obtained from MCF-7 cells were averaged and assigned a value of one to assess relative fold increase in expression in MDA-MB-231 cells.

of PAI-1 [28,29]. The cycling activities of proteolysis and protease inhibition lead to sequential rounds of cell detachment-reattachment, which in turn leads to an increase in cell motility. Indeed, elevated expression levels of both uPA and PAI-1 are characteristic of advanced stage breast cancers [30]. Interestingly, although PN-1 is structurally and functionally related to PAI-1, there have been no studies to date investigating if PN-1 contributes to breast cancer progression in a manner similar to that of PAI-1. To address this gap in knowledge, we examined if PN-1 expression is altered in human breast cancer by quantitating levels of PN-1 expression in human tissue samples obtained from tumor biopsies. In these same samples, we also quantitated PAI-1 and uPA expression levels for direct comparison to PN-1. Our findings indicate that PN-1 expression is elevated in the majority of human breast tumor tissues examined and that its expression levels are directly correlated with increases measured for PAI-1 and uPA. We also found that the highly metastatic, MDA-MB-231 breast cancer line expresses 3.5-fold greater levels of PN-1 compared to the non-tumorgenic, MCF-7 breast cancer cell line. This increase in PN-1 is also correlated to increases seen for PAI-1 and uPA in MDA-MB-231 cells. The elevated expression of all three genes is consistent with our measurements in human breast tumor samples. The significant differences in PN-1 expression between non-tumorigenic MCF-7 cells and highly invasive MDA-MB-231 cells should provide us with a good basis for identifying the mechanism responsible for altered PN-1 expression seen in breast tissues and allow us to examine PN-1 function in the context of elevated PAI-1 and uPA levels. Taken together, these data indicate that PN-1 expression is increased during breast cancer tumorigenesis and may contribute, along with PAI-1, to uPA-mediated tumor cell motility and a more advanced metastatic phenotype.

Although studies in the literature investigating a role for PN-1 in cancer progression are limited, our results complement and extend data presented in a recent report by Buchholz and colleagues [31]. Their study demonstrated that a highly metastatic pancreatic cancer line overexpressed PN-1, while a less metastatic subclone showed little PN-1 expression. The authors also noted that stable PN-1 overexpression in the less metastatic subclone greatly enhanced its local invasiveness in *in vivo* studies. Our studies expand on these observations by demonstrating an increase in PN-1 expression in human breast cancer tissues.

Quantitating expression of PAI-1 and uPA is of high prognostic value for assessing breast cancer survival outcome [24,25]. Numerous independent studies have shown that patients with low levels of PAI-1 and uPA in their primary tumor tissue have a significantly better survival rate than

patients with high levels of either factor alone. Recently, the prognostic value of PAI-1 and uPA has been verified by a pooled analysis consisting of >8,000 breast cancer patients [26]. In light of the overlapping protease specificities of PAI-1 and PN-1 [32], together with the established role of PN-1 in neuronal cell regulation and motility [33,34], we believe it is likely that PN-1 also plays a role in breast cancer progression by contributing to events necessary for increased tumor cell motility. Increased expression of PN-1 by tumor cells may serve to modulate their adhesiveness or motility [35]. Alternatively, tumor cell activity may be influenced by tumor-stromal tissue crosstalk [36]. The breast neoplastic stroma contains a heterogeneous cell population composed of fibroblasts, myofibroblasts, and endothelial cells, which are all known to synthesize and secrete significant amounts of PN-1 [37]. Although it remains to be determined the precise mechanism by which PN-1 contributes to breast cancer tumor progression, the results of the present study establish a rationale for further investigation of PN-1 as a modulator of uPA activity in breast tumor cell motility. Future studies will be focused on identifying transcriptional and/or translational mechanisms controlling PN-1 expression by cancer cells and determining if PN-1 serves as an independent prognostic indicator of breast cancer staging by using a more widely defined sample of tumor tissues, including earlier stage cancers as well as late-stage carcinomas. In addition, the use of laser capture dissection technology will confine our QRT-PCR measurements to tumor tissue and eliminate contributions from surrounding normal mammary tissue that are likely to occur when using surgical specimens.

Conclusion

We quantitated PN-1 expression in samples obtained from biopsies of human breast tumors and from normal mammary tissues by QRT-PCR analysis and compared these results to those obtained for PAI-1 and uPA. Our findings indicate that PN-1 expression is elevated in a majority of human breast tumor tissues examined when compared to normal human mammary tissue. In addition, the elevated PN-1 expression in tumor tissues directly correlates with increased expression measured for PAI-1 and uPA. We also found that the highly metastatic, MDA-MB-231 breast cancer cell line expresses greater levels of PN-1 compared to the non-tumorgenic, MCF-7 breast cancer cell line. Consistent with observations obtained from tumor biopsies, PN-1 expression levels in MDA-MB-231 directly correlate with increases found for PAI-1 and uPA. These data indicate that PN-1 expression is increased during breast cancer tumorigenesis and may contribute, along with PAI-1, to uPA-mediated tumor cell motility and a more advanced metastatic phenotype.

Abbreviations

uPA, urokinase-type plasminogen activator; PAI-1, plasminogen activator inhibitor; ECM, extracellular matrix; PA, plasminogen activation; SERPIN, serine protease inhibitor; PN-1, protease nexin-1.

Competing interests

The author(s) declare that they have no competing interests.

Authors' contributions

BJC carried out the majority of studies and drafted the manuscript. WCH and CMH provided confirmed breast tumor and normal mammary samples. JKG assisted with data interpretation. RAO provided the original conceptual framework for the study, participated in the experimental design and finalized the manuscript for submission. Authors read and approved the final version.

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